CHARLES UNIVERSITY IN PRAGUE

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

Department of Pharmaceutical Chemistry and Drug Control

Chromatography center JSC OlainFarm, Latvia

Forced degradation study of 1-aminohydantoin hydrochloride using HPLC method

DIPLOMA THESIS

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Kamila Benešová

CONTENT

ABSTRAKT

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FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

Katedra farmaceutické chemie a kontroly léčiv

Chromatography center JSC OlainFarm, Latvia

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Název diplomové práce:	Zátěžové zkoušky 1-aminohydantoin-hydrochloridu s využití metody HPLC

Zátěžové zkoušky, rovněž nazývané stresové zkoušky, jsou postupy prováděné s cílem prozkoumat změny v léčivé látce při vystavení nadměrným externím podmínkám. Tato diplomová práce je v teoretické části zaměřena na obsah a charakteristiky zátěžových studií, popis metody HPLC a v experimentální části na studii, která proběhla v JSC OlainFarm s látkou 1-Aminohydantoin-hydrochlorid (AHH). V souvislosti s touto látkou je uváděna známá nečistota 2-(2-karbamoylhydrazinyl)octová kyselina (SAA), což je výchozí materiál a zároveň potenciální degradační produkt. Cílem studie je odhalit chování a degradační cesty AHH při stresových zkouškách.

Nejprve byly vzorky připraveny a vystaveny stresovým podmínkám, které zahrnovaly změny pH, oxidaci, světlo, zvýšenou teplotu, kombinaci zvýšené vlhkosti a teploty. Následně byly vzorky analyzovány předem vyvinutou metodou HPLC, kterou se sledoval pokles účinné látky a případný výskyt nečistot. Získaná data byla porovnána se slepými vzorky, které podstoupily stejné podmínky a se vzorky látky AHH, které nebyly vystaveny stresovým podmínkám.

Výsledky studie ukázaly největší nestabilitu látky za přítomnosti kyseliny a zásady. Po vystavení světlu, zvýšené teplotě a při kombinaci zvýšené vlhkosti a teploty byla látka stabilnější. Při testech na oxidaci se projevila použitá metoda jako nevhodná a tudíž nedostatečně přesná. Přítomnost známé nečistoty SAA byla zaznamenána při působení vyššího pH a při vystavení roztoku látky zvýšené teplotě. Konečný výsledek ukazující stabilitu látky AHH a přítomnost nečistot včetně SAA může být nápomocný při vývoji metod, které budou indikovat stabilitu a monitorovat nečistoty v průběhu následných formálních studií stability a dalších vývojových procesech.

ABSTRACT

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Title:	Forced degradation study of 1-aminohydantoin hydrochloride using HPLC method

Forced degradation, also called stress testing, is a helpful practice carried out in order to explore changes to a drug substance under excessive external stress condition. This thesis focuses on content and characteristics of forced degradation studies and description of the HPLC method in theoretical part and on the study conducted in JSC OlainFarm with the substance 1-Aminohydantoine hydrochloride (AHH) in the experimental part. There is only one known impurity 2-semicarbazidoacetic acid (SAA), which is a starting material and potential product of degradation. The aim of the study is to reveal the behavior and degradation pathways of AHH under stress testing.

First, the samples of AHH were prepared and exposed to stress conditions which included pH, oxidation, light, heat, combination of heat and humidity. Then, they were analyzed by previously developed HPLC method which showed the decrease of the substance and possible presence of impurities. Acquired data were compared with blanks that underwent the same conditions and with unstressed samples.

The study results showed the substance was most unstable in the presence of acid and base. After exposure to light, elevated temperature and at a combination of elevated humidity and temperature, the substance was more stable. During oxidation tests the used method was inappropriate, and therefore insufficiently accurate. The presence of SAA was observed after exposure to base and at elevated temperature of the substance in solution. The final outcome showing stability of AHH and presence of impurities including SAA can be useful for establishment of stability indicating and impurity monitoring methods during subsequent formal stability studies and other developmental processes.

ABBREVIATIONS

AHH	1-Aminohydantoine hydrochloride
AIBN	Azobisisobutyronitrile
API	Active Pharmaceutical Ingredient
EMEA	European Medicines Agency
FDA	U.S Food and Drug Administration
HILIC	Hydrophilic Interaction Chromatography
HPLC	High Performance Liquid Chromatography
ICH	The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IND	Investigational New Drug
IUPAC	International Union of Pure and Applied Chemistry
LC–MS	Liquid chromatography–mass spectrometry
SAA	2-Semicarbazidoacetic acid, 2-(2-carbamoylhydrazinyl)acetic acid

1. INTRODUCTION

ICH defines stress testing of drug substance as “studies undertaken to elucidate the intrinsic stability of the drug substance, part of the development strategy normally carried out under more severe conditions than those used for accelerated testing” (1).

ICH guidelines published the main documents for stability studies. Other authorities such as FDA, EMEA and national authorities provide guidelines based on those from ICH. They give basic information, definitions and characteristics of stress studies that are not strict to follow. They are more of recommendations leaving the specifications for scientists themselves to decide what is suitable for individual drug substance.

The main purpose of stress studying is to show the specificity of stability-indicating and impurity-monitoring methods (2). The analytical method can be proposed as stability-indicating if it is “capable of detecting the loss in content of the active component and subsequent increase in degradation products” (3).

The most important tool to achieve stability-indicating method is HPLC. Major advantages of this method are saving of time, high selectivity and reduced consumption of mobile phase. HPLC is used for identification and quantification of API and its formulations during discovery, development and manufacturing. In the stage of development, HPLC is used to characterize products of the chemical synthesis by analyzing the API, impurities and degradation products generated by accelerated aging.

The HPLC system used for stability-indicating methods development and validation contains 3D data capabilities such as diode-array detectors or mass spectrometers for detection of homogeneity within the chromatographic peaks (4).

2. THEORETICAL PART

2.1 SELECTION OF STRESS CONDITIONS

2.1.1 Photostability

Main document for photostability testing is Q1B: Photostability Testing of New Drug Substances and Products from ICH. It gives information and specifications about procedure, light sources and preparation of samples and it also explains general terms and types of testing. According to this guideline samples of drug substance in solid and solution form or drug product should be exposed to both white and UV light with illumination not lower than 1,2 million lux hours and UV energy needs to be at least 200 watt hours/m^2 light. Samples should be in chemically inert and transparent containers while solid drug samples are recommended to be spread across the container (suitable glass or plastic dish) in a layer with thickness not bigger than 3 mm to provide maximum area of light exposure. The dark control (protected from light with aluminum foil) in the same environment alongside the samples can be used (5). It is suggested that temperature is controlled to prevent temperature changes to the substance. At the end of exposure period the samples can be analyzed for physical properties such as appearance, clarity, color of solution, and for assay and degradants (6). The rate of degradation by light depends on the intensity of incident light and quantity of light absorbed by molecule hence the photosensitivity of the drug involved.

The photolytic degradation can be divided into two types of reaction mechanisms:

1. non-oxidative photolytic reaction includes isomerization, dimerization, cyclization, rearrangements, decarboxylation, hemolytic cleavage of X-C hetero bond, N-alkyl (dealkylation, deamination), SO_2-C bond, etc.
2. oxidative photolytic reaction when $^1O_2$ singlet oxygen reacts with unsaturated bonds (alkenes, dienes, polynuclear aromatic hydrocarbon) to yield photoxidative degradation products or when $^3O_2$ triplet oxygen reacts with free radical of the molecule and creates peroxide

We can see that light can act as catalyst of oxidation reactions (7).

2.1.2 Heat – Thermal stress testing (dry heat, wet heat)

Thermal stress testing is done under more severe conditions than accelerated test conditions as it is recommended by ICH Q1A. The guideline states the effect of temperature should be studied in 10°C increments (e.g. 50°C, 60°C, etc.) above the accelerated testing and humidity at 75% relative humidity or greater (5). Solid-state drug substances should be exposed to dry and wet heat. Liquid drug products are to be exposed to dry heat (6). Rate of reaction changes with higher temperatures as it is demonstrated by Arrhenius equation: $\mathbf{K=Ae^{-Ea/RT}}$. Where K is specific reaction rate, A is frequency factor, Ea energy of activation, R gas constant and T absolute temperature. Hence the drugs are susceptible to degradation if we increase the temperature. Thermal degradation involves different mechanisms such as pyrolysis, hydrolysis, decarboxylation, isomerization, rearrangement and polymerization (7).

2.1.3 Acid and base hydrolysis

Hydrolysis is a solvolytic process in which the solvent involved is a water. It is a chemical degradation reaction when drug substance reacts with this solvent resulting in breakdown products with different chemical structure. Adding acid or base to the reactions cause catalyzation of ionisable functional groups present in the molecule (7).

The most suitable reagents used in stress studies are hydrochloric acid or sulfuric acid (0,1 -1M) for acid hydrolysis and sodium hydroxide or potassium hydroxide (0,1 -1M) for base hydrolysis. The recommended strategy for hydrolysis suggests first reactions to be done in various pH (e.g. 2-7, 10-12) at room temperature for two weeks or up to 15% degradation (6). If we have a new drug we can start refluxing it in 0,1 M HCl / 0,1 M NaOH. After seeing reasonable degradation the testing can be stopped. If there is not any degradation seen we can use higher strength of acid or base or longer duration of reaction time (7).

For lipophilic drugs co-solvents may be needed to help solubilizing the drug substances. They should not contain functional groups prone to reactions in a molecule such as methanol.

Samples should be analyzed in various time intervals to see progress of degradations and to distinguish between primary and secondary degradants (6).

2.1.4 Oxidation

Many drug substances undergo autoxidation. It is oxidation that happens in presence of ground state elemental oxygen under normal storage conditions. It requires a free radical initiator to begin the chain reaction. The mechanism involves electron transfer to form reactive anions and cations. Amine, sulphide and phenol give N-oxide, hydroxylamine, sulphone and sulphoxide in these reactions. The functional group with labile hydrogen like benzylic carbon, allylic carbon and tertiary carbon or α-positions of hetero atoms are susceptible to oxidation to form hydroperoxide, hydroxide or ketone (7).

Hydrogen peroxide is used most often because it is the closest to possible presence of peroxides in excipients. Metal ions, oxygen, radical initiators (AIBN) can be also used. The choice of the suitable oxidizing agent and its concentration and conditions depends on drug substance. There is no guideline that would give a close specifications. Some sources suggest subjecting the solution to 0, 1 - 3% hydrogen peroxide at neutral pH or room temperature for seven days or up to 20% degradation (6). Other source recommends 3-30% concentration of hydrogen peroxide at temperature under 40°C for 2-8 days while initially 3% H_2O_2 is used at room temperature for 6 hours and then it is increased or decreased to generate relevant degradation products. According to this source the time can be changed to only 24 hours with 30% or decreased up to 30 min with 1% H_2O_2 (7).

2.2 REGULATORY CONSIDERATIONS

Stress testing is a part of stability section in application for regulatory authorities. It is a part of development strategy. It is required to have all significant impurities from stress studies identified, qualified and quantified and those should be reported during the Phase III of IND. However, it is highly recommended to start stress testing before Phase II and have it completed for the Phase III to improve manufacturing, optimal choice of suitable analytical techniques for stability and assure sufficient time and optimization of stress conditions. And also because any change in manufacturing or stability-indicating analytical methods requires re-validating. Method validation must be completed before starting long-term stability studies.

Forced degradation is important component of validating analytical methods that are stability-indicating and impurity-monitoring. What they focus on can be seen in Table 1 (2).

Stability-indicating methods	Impurity-monitoring methods
Which parameters are the best indicators of product stability?	detection of potential degradation products
What kind of structural transformation happens?	detection of unrelated impurities and product-related impurities
Can accidental exposure (for example during transportation) influence the product?	separation of product-related degradants and those derived from excipients and placebo (product-related impurities are formed during manufacture, storage, use – they have different properties with respect to activity, efficacy, safety)
	elucidation of degradation pathways
	identification of degradation products that may generate during storage and use – improvements for manufacturing process

Table 1: Analytical methods

2.3 EXTENT OF DEGRADATION

It is recommended to have degradation level in between 10 and 15% for validation of purity assay. Some other authors state that degradation level should be less than 10%. Reynolds (8) and some others see spiking of samples with mixture of known degradation products as a good option. Carr and Wahlich suggest that if the expected range of impurities is 0-10% it should be spiked with impurities by 0-20%. General rule by scientist is that the degradation optimal for analytical method validation should be around 10%, because small organic pharmaceutical drugs have typical limit on a label 90% (2).

If the stress conditions are not strong enough they are useless however the more extreme stress conditions are the more they can lead to secondary degradation products that would not appear in formal stability studies. That is why it is important to achieve purposeful degradation that would be predictive of long-term and accelerated storage conditions. Generally recommended is 5-20%, this range covers 10% which is the most common limit 90-110% of the label claim of small molecules (6).

2.4 IMPURITIES IN DRUG SUBSTANCES

Recently not only purity profile but also impurity profile has become essential part of the information about chemistry, manufacturing and control (CMC) provided to regulatory authorities (7). Impurity profile is a description of the identified and unidentified impurities present in a new drug substance. Unidentified impurity is impurity for which a structural characterization has not been achieved and that is defined solely by qualitative analytical properties (e.g. chromatographic retention time) (9).

ICH defines impurity as "any component of the new drug substance that is not the chemical entity defined as the new drug substance. ". According to ICH guidelines Q3A (R2) every impurity should be identified with respect to two perspectives. What they include can be seen in Table 2.

Chemistry Aspects	Safety Aspects
classification and identification of impurities	specific guidance for qualifying absent impurities
report generation	specific guidance for qualifying those impurities that were present at substantially lower levels
listing of impurities in specifications	on batches that are used in safety and clinical studies
brief discussion of analytical procedures	

Table 2: Impurities in new drug substances

Impurities can originate during synthesis, form during degradation or reactions between drug substances and excipients. They can be either process related (PRI) which include starting material (material used in the synthesis of a new drug substance that is incorporated as an element into the structure of an intermediate and/or of the new drug substance, it is normally commercially available and of defined chemical and physical properties and structure), reaction intermediate, chemical reagent, ligand, catalyst, by-product of synthetic route, residual solvent or degradation related (DRI) due to hydrolysis, oxidation or photolysis of a drug.

The ICH (guideline Q3A R2) (9) divides impurities into three categories: organic, inorganic, residual solvents. Organic impurities include starting materials, by-products, intermediates, degradation products, reagents, ligands and catalysts. They can be identified or non-identified, volatile or non-volatile. They start appearing during manufacturing process or the storage. Inorganic impurities include reagents, ligands and catalysts, heavy metals or other residual metals, inorganic salts, other materials (e.g., filter aids, charcoal). They are usually known, identified, detected and quantified by pharmacopoeial procedures. They appear during manufacturing process. Residual solvents are inorganic or organic liquids used as vehicles for the preparation of solutions or suspensions in the synthesis of a new drug substance. They appear during production of API or manufacturing. Guidelines for residual solvents Q3C (R5) (10) divide residual solvents into three categories based on their risk to human health:

Class 1: Solvents to be avoided (e.g. benzene; carbon tetrachloride; 1,2-dichloroethane; 1,1-dichloroethene)

Class 2: Solvents to be limited (e.g. methanol, acetonitrile, chlorobenzene, pyridine, toluene)

Class 3: Solvents with low toxic potential (e.g. acetone, acetic acid, butanol, ethanol)

2.5 ANALYTICAL PROCEDURES

Organic impurity levels can be measured by a variety of techniques, including those that compare an analytical response for an impurity to that of an appropriate reference standard or to the response of the new drug substance itself. Reference standards used in the analytical procedures for control of impurities should be evaluated and characterized according to their intended uses. The drug substance can be used as a standard to estimate the levels of impurities (9).

Stability indicating methods may include various analytical procedures such as electrophoresis or high-resolution chromatography (reversed phase chromatography, gel filtration, ion exchange, and affinity chromatography).

Conventional methods (column chromatography) or hyphenated techniques (LC–MS, LC–nuclear magnetic resonance) can be used in the identification and characterization of the degradation products. New analytical technologies using various chromatographic techniques like reversed phase high performance liquid chromatography, thin layer chromatography or gas chromatography can also be used to separate and analyze unknown impurity. HPLC-photodiode array ultraviolet detector (DAD) and LC–MS can be used when degradants cannot be isolated in pure form and also to compare the relative retention time, UV spectra, and mass spectra (11).

2.6 CHROMATOGRAPHY

Chromatographic methods are separation methods based on different distribution of divided substances in mixture between two different immiscible phases: mobile and stationary. In liquid chromatography mobile phase consists of liquid, stationary phase is either solid or liquid adsorbed in a solid carrier. Stationary phase in chromatographic system is in form of sorbent, mobile phase flows through this sorbent. Most common method of chromatography is HPLC (High Performance Liquid Chromatography). In this case the mobile phase is supplied to the system with help of pump in high pressure.

2.6.1 HPLC instrumentation

As can be seen in *Figure 1* liquid chromatograph consists of following parts:

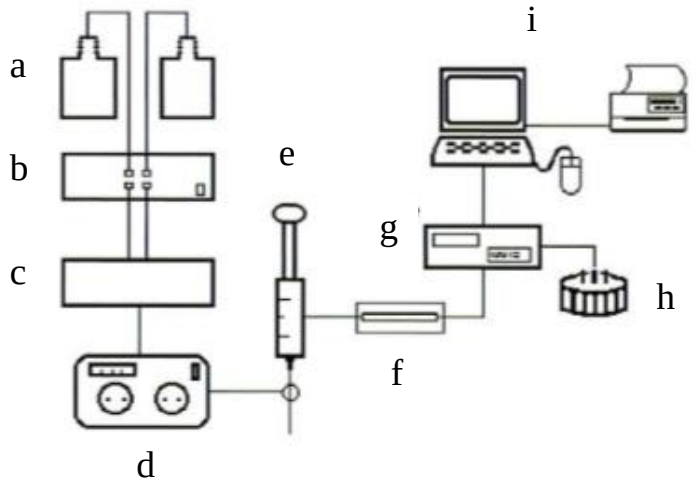


Figure 1: Block diagram of HPLC: (a) reservoirs of mobile phase, (b) degasser, (c) mixer, (d) high-pressure pump, (e) equipment for sample injection, (f) column, (g) detector, (h) fraction collector, (i) computer

(a) reservoirs of mobile phase

Reservoirs are used for storage of mobile phase. Depending on the type of elution, there can be one reservoir from which mobile phase is flowing to high-pressure pump (d). This is used when there is no change of concentration of mobile phase happening *(isocratic elution).* There can also be more reservoirs or more pumps in the system *(also for gradient elution).* In this case mobile phase is brought from reservoirs to mixer (c) where it is mixed.

(b) degasser

Degasser is the part of chromatograph responsible for degassing of mobile phase in order to decrease creation of small bubbles from dissolved gas created by change of pressure. There are two principles of degassing - helium or vacuum degasser.

(c) mixer

(d) high-pressure pump

Pump transports mobile phase through column to detector by piston or membrane. The pressure of pump has to be in level of 1-100 MPa. There can also be two pumps in front of mixer in case of gradient elution.

(e) equipment for sample injection

There are two types of equipment for sample injections - high pressure manual valve (30-80 MPa) or autosampler. Autosampler is connected to reservoir of samples with vials.

(f) column

Separation of substances takes place in this location. The inner surface should be stable in high pressure (100MPa), chemically stable in presence of mobile phase and it should be smooth. It consists of the body and ending. The body is straight tube with smooth inner surface. The ending has three functions. It is to make sure the column is closed tightly, distribution of mobile phase and sample is the same in all parts of column and it holds the content of column.

(g) detector

After flowing through column the sample goes inside detector where it is detected (12). The detector translates the changes of the column effluent into an electrical signal which can then be processed to obtain the required information. The choice of the detector depends on the chemical properties of the analytes, the desired range of sample concentrations and on the chromatographic system. The higher the quality of the detection in terms of selectivity and sensitivity, the less demands are put on the separation system and vice versa (13).

(h) fraction collector

Fraction collector is mainly used in preparative chromatography.

(i) computer

Computer with specific software is used to collect data from detector for subsequent evaluation.

2.6.2 Stationary phase

Stationary phase is a part of chromatographic system which consists of solid or film of liquid absorbed or chemically connected to solid carrier. The most important properties are chemical and thermal stability, no interactions with mobile phase and it should not be dissolving in mobile phase. Stationary phases can be divided to groups by chemical composition:

1. Inorganic oxides (silica gel, zirconium oxide, alumina and titanium dioxide)
2. Chemically bonded silica stationary phases
3. Polymeric stationary phases
4. Hybrid silica stationary phases
5. Stationary phases based on graphitic carbon

Silica gel

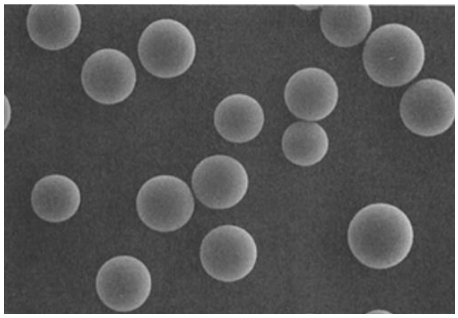


Figure 2: Spherical silica gel with purity over 99,99% (29)

Silica gel is the most common polar inorganic sorbent. After chemical binding of ligands it can be used for reversed chromatography. Alone it is used for separation on normal phases and HILIC. It is highly resistant to high pressures. The most common is porous amorphous form $SiO_2.xH_2O$. The water is chemically bonded to form silanol groups Si-OH. The active centers on surface of silica gel are hydroxyl groups. There are more types of them: isolated, vicinal, geminal or they can be connected by hydrogen bound. They show different acidity and polar character and they can be used for preparation of chemically bonded silica stationary phases. In high temperatures the double groups and groups connected by hydrogen are removed and simple isolated groups are stabilized. The water which covers the virgin silica gel can be removed by heating to 150°C which leads to activation of silica gel. Heating to 300 – 500 °C leads to condensation of neighboring OH groups to siloxane groups. Higher temperatures cause cleavage of free OH groups hence creates hydrophobic properties.

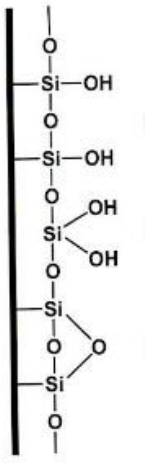


Figure 3: Silanol groups on surface of silica gel: vicinal silanol, geminal silanol, siloxane groups

Silica gel starts dissolving in mobile phase with pH 8 so it is recommended to use only for pH 7 and lower.

Chemically bonded silica stationary phases

Creation of these phases has a lot of advantages, such as almost no elution of stationary phase from the carrier, it does not dissolve in mobile phase, no mechanical entrainment at high mobile phase flow, robustness to change the temperature and composition of the mobile phase. Thanks to these parameters there is no unbalance, there can be different work conditions (composition of mobile phase, change of temperature).

The examples of reactions can be seen in Figure 4 and Figure 5.

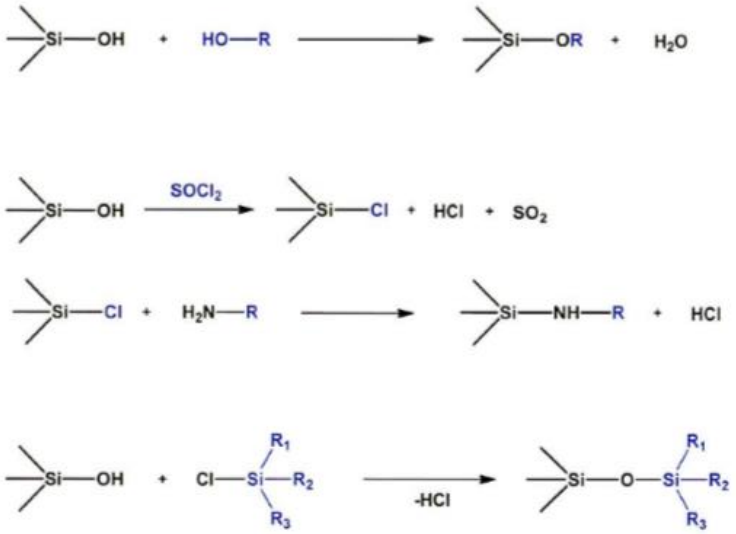


Figure 4: Chemical modification of silica gel

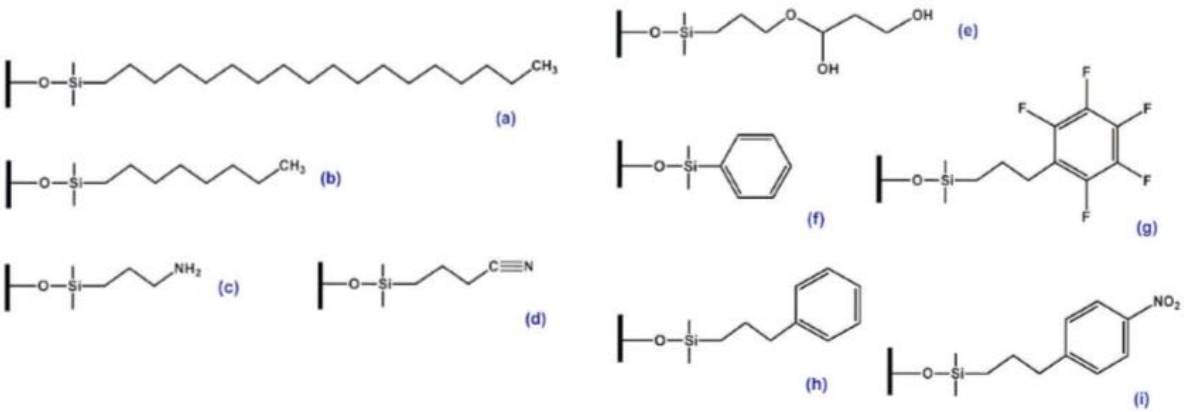


Figure 5: Chemically bonded silica stationary phases: (a) C_{18} ODS, (b) C_8, (c) propylamine phase, (d) propylcyan phase, (e) diol phase, (f) phenyl phase, (g) pentafluorphenyl, (h) propylphenyl, (i) propylnitrophenyl

2.6.3 Chromatographic systems

Brief explanation and description below is directed to only some types of chromatographic systems. The other chromatographic systems not listed below are mixed-mode chromatography, paired-ion chromatography, micellar liquid chromatography, micellar electrokinetic chromatography, affinity chromatography, hydrophobic interaction chromatography (HIC), and chiral chromatography.

Normal Phase (NP-HPLC)

Stationary Phase	Mobile Phase
Polar	Non-polar
most common is pure silica gel or chemically bonded silica phases with following groups: diol group, nitro group (slightly polar) cyano, cyanopropyl (moderately polar) short aliphatic amino groups (strongly polar)	mostly binary mixtures of solvents of different polarity (e.g. aliphatic hydrocarbon and propanol) non- polar part: *pentane, hexane, toluene, benzene, chloroform, acetonitrile* polar part: *propanol, ethanol, methanol, ethylene glycol*

Table 3: Characteristics of Normal Phase HPLC method

Stationary phases used in normal phase chromatography systems need to be polar. Mobile phases are characterized by lower polarity than stationary phases. Normal phase chromatography is based on competition between sample and mobile phase for adsorption spectra on surface of stationary phase (e.g. free hydroxyl groups of silica gel). Retention of substances on column grows with growing polarity of analytes and it decreases with bigger concentration of polar solvent in mobile phase. Specific interaction of substance-adsorbent is mainly hydrogen bound or they have electrostatic character. There are also steric effects applicable (stronger retention of cis isomers). Large functional groups which sterically shade the polar group in molecule are also reason of lower retention.

The disadvantage is the content of water in mobile phase. The water is absorbed on active places of surface which leads to decreased retention volumes of separated substances. It is recommended to use closed system with humidity control (precolumn with alumina or silica gel with a known water content).

Normal phase chromatography is currently retreading because of disadvantages compared to separations in other systems (non-aqueous, volatile organic solvents). It is however used in systems which decompose or dissolve in presence of water (lipophilic substances) and for separation of isomers (tocopherols, retinols, retinals) (12).

Reversed-Phase (RP-HPLC)

IUPAC (14) explains reversed-phase chromatography as "an elution procedure used in liquid chromatography in which the mobile phase is significantly more polar then the stationary phase."

Stationary phase	Mobile phase
Non polar	Polar
long carbon chains on the surface of the carrier (silica gel, hybrid sorbent or other metal oxides) C_{18} is the most common *propylamine and propylcyano* - depending on the mobile phase normal or reversed system (most on Figure 5)	Usually mixture of aqueous ingredients (*water, dilute aqueous solutions of acids or bases, buffers*) with polar organic solvents which are miscible with water (*alcohols, acetonitrile, tetrahydrofuran*)

Table 4: Characteristics of Reversed-Phase HPLC method

There are no polar interactions, only weak dispersion forces i.e. non-specific molecular interactions between the analyte-adsorbent. These interactions are much weaker. Retention depends on non-polar phase (the length and number of the alkyl, a plurality of aromatic rings or large substituents such as halogens), but mainly on composition of mobile phase. Balance is established faster than normal phase, because there is no influence by residual water. The choice of solvents is smaller and limited to only mixtures of water-organic solvent. The water alone cannot be used because hydrophobic surface of stationary phase is not easily wettable by water and may lead to hydrophobic collapse (12).

Hydrophilic interaction chromatography (HILIC)

HILIC is a separation technique with aqueous mobile phase which consists of water or buffer with organic solvent especially acetonitrile thanks to its low viscosity, high separation efficiency and low pressure to the chromatographic systems. Stationary phase is highly hydrophilic (15).

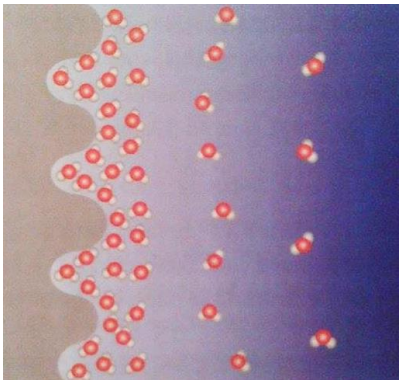


Figure 6: HILIC: Layer of molecules of water (red) on surface of polar stationary phase (grey) (12)

Stationary phases for HILIC can be

- neutral - without electrostatic interactions (diol, amide, cyclodextrin)
- charged - strong electrostatic interactions (silica gel, aminopropyl silica gel)
- zwitterions (molecules with both positive and negative electrical charges) - low electrostatic interactions (sulfobetaine, ZIC-HILIC)

As can be seen on Figure 6 there is absorption of water on polar stationary phase which leads to formation of the diffusion layer with concentration gradient of water towards the mobile phase.

In HILIC systems there is a high influence of sample diluent. Sample should be dissolved in the same or similar diluent to the mobile phase (12).

To sum up HILIC actually combines three separation techniques as can be seen in Figure 7 – reversed phase chromatography for mobile phase, stationary phase is similar to normal phase chromatography, and the compounds that can be analyzed are the same as the ones for ion exchange chromatography (charged compounds) (16).

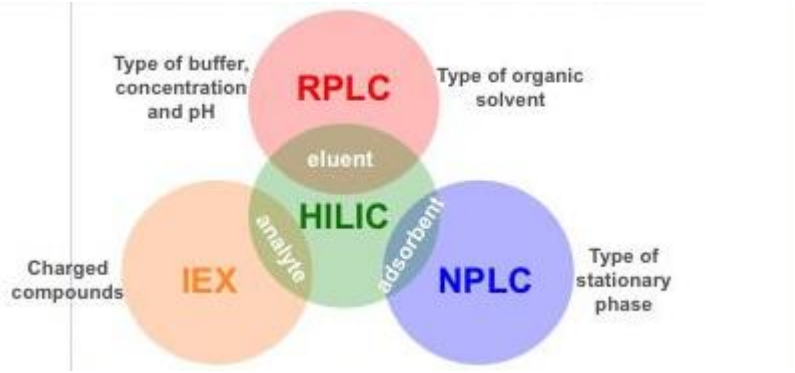


Figure 7: HILIC as combination of three methods (16)

HILIC is mostly used for analysis of polar and hydrophilic substances such as carbohydrates, amino acids, polar organic acids and bases. Retention is increased with higher polarity of substance and decreased with higher polarity of mobile phase (17).

Ion exchange chromatography (IEC)

IEC is the oldest chromatographic technique with the liquid phase. For IEC there are typical strong electrostatic interactions between the ionized functional groups of transducers (stationary phase) and the oppositely charged ions of the surrounding solution. Strength of the interaction is determined by the number and size of the charge of the analyte and functional groups. IEC is mainly used for determination of amino acids, separation of cations and anions in water, separation of carboxylic acids in wine, nucleic acids and peptides.

Size exclusion chromatography (SEC)

SEC also known as **gel permeation chromatography** (GPC) is based on distribution of molecules according to their size in the column. There are no other interactions happening. The stationary phase consists of porous polymer gel and the mechanism of separation is different penetration of individual molecules of solute into the pores of the gel particles. Large molecules do not enter the gel pores due to steric constraints and they will pass through the column, in particular through the external space between the particles completely filled with liquid. Small molecules will penetrate the pores according to their size and the size of available pores so their release from column will delay. Large molecules are eluted earlier than the small ones which have to travel much longer distance in the column (12).

2.6.4 Types of detectors

Spectrophotometric detectors are based on absorption of light in 190 – 800 nm according to the Lambert-Beer law. The disadvantage can be that the detector shows difference when the mixture goes through change of chemical balance (ion or molecule interactions, dissociations, association, dimerization, polymerization, creation of complexes, hydrolysis). This type of detector can have fixed wavelength (most common 253,7 nm) or it can be changed to predetermined wavelength or programmable in some range and measured at more wavelengths at the same time. This type is less sensitive. The most modern type is photodiode-array detector (PDA). It measures the whole spectrum of wavelengths in real time without stopping the separation (12). The full polychromatic light of the lamp passes through the optical cell before it is dispersed on a grating. The dispersed lights strikes an array of light-sensitive diodes. Each diode corresponds to a specific wavelength and it needs to be recharged after every measuring cycle. The amount of electric charge needed is used as a measure of the light intensity which is converted into absorbance values (13). PDA enables the detection of substance in any wavelength. It is connected to computer and can be used to compare spectra with library and calculate the purity of peak which can show if there is only one substance eluted in peak or if it is a mixture of undivided analytes.

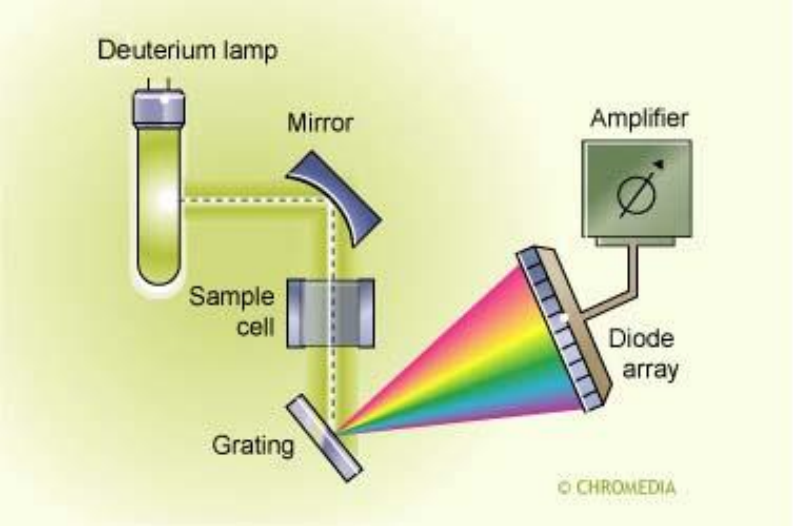


Figure 8: Diode Array Detector UV *(15)*

Fluorescent detectors are used when detecting the substance that exhibit fluorescence or they can be transformed to fluorescent derivatives after reaction with suitable agents. This method is less versatile, but it is more sensitive and it can be used during gradient elution.

Electrochemical detectors are used for substances connected with the action of electrochemical reaction happening on phase boundary electrode-eluent. The ability of reduction and oxidation can be used with amperometric or coulometric detector (12).

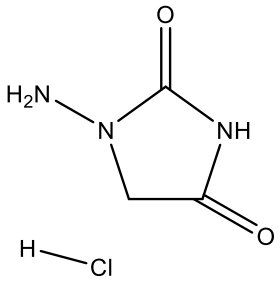
Mass spectrometer is a type of detector that can be connected to HPLC. The eluent is freed of mobile phase and the molecules of substance in gas state are ionized by electron impact, heat or by electrical ionization. Charged particles are separated according to their mass and charge in the magnetic high frequency field and the mass spectrum is recorded. The mass spectrum shows frequency of ions in relation to a weight / number of charges. This method is highly selective and sensitive but it is financially difficult method (18).

Refractive index detector compares refractive index of eluate and mobile phase. It is less sensitive, there is temperature dependence, importance of constant flow of mobile phase and it is not possible to use gradient elution in this case (12).

2.7 INFORMATION ABOUT SUBSTANCE AND IMPURITIES

AHH

1-Aminohydantoin hydrochloride



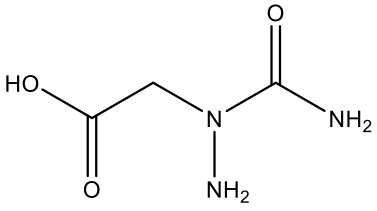
(19)

CAS Registry Number:	2827-56-7
Chemical Name:	1-amino-2,4-imidazolidinedione hydrochloride
	1-aminohydantoin hydrochloride
	AHD.HCl
	1-Aminohydantoin Hydrochlorid
	1-aminoimidazolidine-2,4-dione hydrochloride
	1-Amino-imidazolidin-2,4-dion Hydrochlorid
	1-aminohydantoin-hydrochloride
Molecular formula:	$C_3H_5N_3O_2.ClH$
Molecular Weight:	151.553
Melting Point (Experimental):	201-203 °C
Type of Substance:	heterocyclic

(20)

SAA

2-[aminocarbonyl(azanyl)amino]ethanoic acid



(19)

One known impurity of 1-Aminohydantoin hydrochloride is 2-Semicarbazidoacetic acid, which is a starting material and potential product of degradation of 1-Aminohydantoin hydrochloride.

PubChem Compound ID:	67308
Molecular Formula:	$C_3H_7N_3O_3$
Molecular Weight:	133.10598 [g/mol]
Hydrogen Bond Donor Count:	3
Hydrogen Bond Acceptor Count:	4
Rotatable Bond Count:	2 (21)

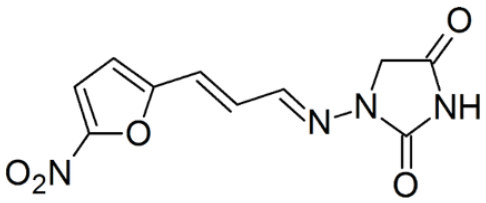
Name of structure according to ChemDraw: N-amino-N-carbamoylglycine

Chemical properties from ChemDraw:

Boiling Point:	625,12 [K]
Melting Point:	532,59 [K]
Critical Temp:	809,19 [K]
Critical Pres:	64,83 [Bar]
Critical Vol:	309,5 [cm3/mol]
Gibbs Energy:	-254,77 [kJ/mol]
Log P:	-2,03
MR:	28,82 [cm3/mol]
Henry's Law:	8,5
Heat of Form:	-432,99 [kJ/mol]
tPSA:	109.65
CLogP:	-1.53058
CMR:	2.8994
LogS:	1.113
pKa:	3.717 (19)

FURAZIDIN (FURAGIN)

1-{[(1E,2E)-3-(5-Nitrofuran-2-yl)prop-2-en-1-ylidene]amino}imidazolidine-2,4-dione



CAS: 1672-88-4

ATC code: J01XE03 (22)

Chemical names: FURAGIN; Furazidine; Furazidin; 1672-88-4; Akritoin (21)

AHH is a starting material for Furazidin (also called Furagin) which is a nitrofuran derivative used to treat antibacterial infections. It has been manufactured and distributed under brand names such as Furamag, Furasol, Urofuraginum in Poland, Georgia, Russia and Latvia (23).

Furagin is used for the same indications (mainly for acute bacterial cystitis) as the more popular 5-nitrofuran derivative Nitrofurantoin. Both substances have very similar pharmacokinetic properties and the same resistance profile (24).

Therapeutic indications of Furamag include treatment of urinary tract infections (cystitis, urethritis, and pyelonephritis), prevention of the reoccurrence of urinary tract infections and for prevention in patients with urinary catheters or before and after urological surgeries. It is also used for treatment of prostatitis and gynecological infections (25). Information about pharmacology from description of medicine Furamag from Latvian Register of Medicinal Products (26) can be seen in Table below.

Pharmacodynamic properties	**Pharmacokinetic properties**
Spectra	**Absorption and distribution**
Broad antimicrobial spectrum, which includes both gram-positive and gram-negative microorganisms. Compared to other nitrofuran compounds it is most active against staphylococci and many other pathogenic or conditionally pathogenic bacteria (Escherichia coli, Enterococcus faecalis); also active against certain Gram-negative bacteria, including Enterobacter, Neisseria, Salmonella, Shigella.	After oral administration the medication is well absorbed from the gastrointestinal tract, mainly by passive diffusion from the distal segment of the small intestine. Food promotes the absorption of medicine. Following a single oral dose of Furamag 50 mg peak plasma concentrations are reached in approximately 3 hours and last 6 to 8 hours, they can be detected in urine after 2 to 4 hours. Active substance is present in high concentration in lymph. Concentrations in the urine and bile are many times higher than in the serum.
Mechanism of action	**Excretion**
The inhibition of microbial enzyme system and other biochemical processes in the cells of microorganisms which causes the bacterial cell wall or cytoplasmic membrane disintegration.	Excretion is carried out by the kidney tubular secretion, glomerular filtration and reabsorption. Acidic urine ($pH \leq 5.5$) increases exposure and risk of toxicity. Alkaline urine weakens the effect of the drug. The daily dose is excreted from the body within 24 hours.

Table 5: Pharmacology of Furamag

3. THE AIM OF THE STUDY

The main purpose of this study is to investigate the effects of excessive stress conditions on the substance AHH, which is a starting material of drug product Furagin that is used in treatment of bacterial infections. The study was aimed at developing stability-indicating method therefore answering questions such as which parameters are the best indicators of stability and what conditions may influence the substance. This can be used in further developmental processes and during formal stability studies. The next aim of study is to determine sufficient impurity-monitoring method that would help successfully detect potential degradation products, unrelated impurities and product-related impurities, elucidate degradation pathways, separate product-related degradants from those derived from excipients and placebo and identify degradation products that may generate during storage and use for improvements during manufacturing processes.

The study was evaluated using the HPLC method which was previously developed at the laboratory. For the calculation of degradation product was used the normalization method which revealed the content in % of sum of all peaks in chromatogram.

4. EXPERIMENTAL PART

4.1 METHOD

Equipment

- Waters Alliance 2690 separation module equipped with photodiode array detector (Waters 2996), quaternary pump, degasser, column thermostat, automatic sampler and chromatographic software Empower
- Suntest XLS Tabletop Sunlight Exposure System with SunCool Modul, (Atlas Material Testing Technology) GmbH, wavelength range 300-800 nm, xenon lamp
- Humidity Chamber HPC 108, (Memmert), temperature range (with humidity): 20°C to 90°C, accuracy 0.1°C; humidity range 10 to 98 rh%, accuracy 1 rh%,
- Analytical balances, accuracy not less than 0.00002 g.
- Balances, accuracy not less than 0.01 g.
- pH Meter, resolution 0.02.
- Volumetric flasks, amber glass, capacity 20 mL, 25 mL, 50 mL, 100 mL and 1000 mL.
- Volumetric pipettes, capacity 1 mL, 4 mL, 5mL and 10 mL.
- Graduated cylinders, capacity 50 mL, 100 mL, 250 mL and 1000 mL
- Headspace amber vials, amber glass, Supelco, 40 mL
- Durapore® Membrane Filters, pore size 0.22 µm.
- Syringe filters, Glass Fiber, pore size 1.0 µm, diameter 25 mm.
- Syringe filters, PTFE, pore size 0.22 µm, diameter 13 mm.

Reagents

- Methanol, HPLC gradient grade (assay 99.99% (Scharlau, batch No. 15837913)).
- Acetonitrile, HPLC gradient grade (Fisher, batch No. 1417570)
- N,N-dimethylformamide, HPLC grade. (Scharlau, batch No. 15913107).
- Ethanol, 96.2% (AS “Olainfarm”, batch No. 120614).
- Purified water for HPLC. (batch No. 0215(1)D)
- Ammonium acetate, 98%, for analysis. (Scharlau, batch No. 13812901).
- Acetic acid, 99.8 %, for analysis. (Scharlau, batch No. 14717004).
- Hydrochloric acid, 37%, (Scharlau, batch No. 13409309).
- Hydrogen peroxide, 30%wt solution in water, (Acros, batch No. A0345842).
- Sodium hydroxide, assay min. 99.0% (Scharlau, batch No. 14916202).
- Dimethyl sulfoxide (DMSO), assay min. 99.9% (Scharlau, batch No. 13555205).

Materials

1-Aminohydantoine hydrochloride (1-AHH), Lot No……

Reference standards

- 1-Aminohydantoine hydrochloride (1-AHH), IRS, doc. NQS8.214.021 (Olainfarm).
- Semicarbazide acetic acid (SAA),WS, doc.NQS8.150.010 (Olainfarm), purity 97,5%

4.2 HPLC ANALYSIS CONDITIONS

HPLC method with diode array detection used for this forced degradation study of AHH has been previously developed and validated in laboratory at the OlainFarm factory. All conditions were strictly followed without any changes during the process.

Column:	ZIC HILIC, 250 x 4,6 mm, 5 μm
Column temperature:	30 °C
Mobile phase:	10 mM ammonium acetate buffer (pH 4.5) : acetonitrile (20:80,v/v)
Flow rate:	0.5 mL/min
Detector:	UV at 200nm
Injection volume:	5 μl
Run time:	25 min.

Approximate retention time (RT) and relative retention time (RRT) of compounds

Analyte	RT, min	RRT
Blank peak 1	about 5.4	0.6
Blank peak 2	about 6.3	0.7
Blank peak 3	about 6.9	0.8
AHH	about 8.6	1.0
SAA	about 20.5	2.4

Injection sequence and system suitability (SST)

The sequence of injections for analysis is as follows:

1. blank (diluent)
2. resolution solution for estimation SST

Evaluate the following parameters for SST:

There should not be any interfering peak at the retention time of AHH and SAA in the chromatogram obtained from blank. The resolution (Rs) between peak from blank and AHH peak in the chromatogram of resolution solution should not be less than 2.5.

From each vial there was performed one injection for SST.

If SST passed, inject forced degradation sequence:

1. Blank solution (diluent)
2. Control solution (stressed diluent)
3. Resolution solution
4. Reference solution (0.2 mg/ml in diluent) for calculation of mass balance
5. Test solution
6. Blank solution (diluent)

There was performed one injection from each vial for forced degradation analysis of the samples.

4.3 CONDITIONS OF STRESS TESTING

Type of stress testing and equipment	*Conditions*	*Time*
Acid hydrolysis 1 mg/ml closed head space vial	1 M HCl 0,1 M HCl at ambient temperature	1 h 2,5 h 21 h
Base hydrolysis 1 mg/ml closed head space vial	0,1 M NaOH at ambient temperature	3 h 6 h 24 h
Oxidation 1 mg/ml closed head space vial	30% H_2O_2 at ambient temperature	24 h 7 days
Photolytic Suntest XLS Tabletop Sunlight Exposure System with SunCool Modul	***Solid***	2 x the ICH exposure length
	Solution 1 mg/ml AHH in water in transparent glass with air	
	Solution water in transparent glass with air (stressed diluent)	
	Solution 1 mg/ml AHH in water in transparent glass with N_2	
	Solution water in transparent glass with N_2	
Thermal hydrolysis	***Solution 1 mg/ml in water at 50ºC in*** closed head space vial closed head space vial with N_2	24 hours 7 days 14 days 21 days
	Solid at 50°C opened head space vial closed head space vial with N_2	7 days 14 days
Thermal / Humidity	***Solid at 50ºC / 75% RH*** opened vial	7 days 14 days

4.4 PREPARATION OF SOLUTIONS

Acetic acid solution

Transfer 20 mL of acetic acid in to 200 mL volumetric flask, dilute up to the mark with water and mix.

Ammonium acetate buffer (ph 4.5 ± 0.1), 10 mM

Accurately weigh 0.77 g ammonium acetate into glass beaker, add approximately 500 mL purified water and 5 mL acetic acid solution, mix until dissolved, and adjust the pH to 4.5 (±0.1) using acetic acid solution. Transfer ammonium acetate buffer to a 1000 mL volumetric flasks, dilute with water up to the mark and mix.

Filter solution through a filter having porosity of 0.22 μm.

Mobile phase (10 mM ammonium acetate buffer) : acetonitrile (20:80, v/v)

Mix ammonium acetate buffer and acetonitrile in parts 20:80, v/v.

According to the information from laboratory mobile phase is stable for 24 hours.

Sample diluent

Mobile phase

Blank solution

Mobile phase

Reference solution

Place 50 mg of 1-AHH into a 50 mL volumetric flask, dissolve in approximately of 30 mL diluent, sonicate for 10 minutes, allow solution to cool to the room temperature, add the diluent up to the mark and mix. Transfer 5.0 mL of this solution into 25 mL volumetric flask, dilute with diluent up to the mark and mix. *The concentration of this solution is 0.2 mg/mL.*

Resolution solution

Place 50 mg of SAA into a 50 mL volumetric flask, dissolve in approximately 30 mL of ammonium acetate buffer solution, sonicate for 10 minutes, allow solution to cool to the room temperature, add the same solution up to the mark and mix. Transfer 5.0 mL of this solution into 25 mL volumetric flask, dilute with diluent up to the mark and mix. *The concentration of this solution is 0.2 mg/mL (SAA solution).*

Transfer 5.0 mL of SAA solution and 5.0 mL of Reference solution into 100 mL volumetric flask, dilute with diluent up to the mark and mix. *The concentration of this solution is 0.01 mg/mL for each component (or 5% of 1-AHH in the test solution)*

4.5 SAMPLE PREPARATIONS

4.5.1 Acid hydrolysis

Place 0.050 g of 1-AHH into a 50 mL volumetric flask, dissolve in the 40 mL of HCl, sonicate for 10 minutes, allow solution to cool to the room temperature, add HCl up to the mark and mix. *The concentration of solution is 1.0 mg/mL. (1-AHH stock solution AH)*

Transfer into the head space vials and close them. Keep the one sample at ambient temperature and analyze by HPLC in 1 hour; 2,5 hours and 21 hours.

After the expiry of the exposure take corresponding sample of 1-AHH stock solution and transfer 4 mL into 20 mL volumetric flask, dilute with sample diluent up to the mark and mix. *The concentration of this solution is 0.20 mg/mL.* Filter solution through a syringe filters with pore size diameter of 0.22 μm, discarding first few ml of the filtrate (1-AHH test solution AH).

Note: The stressed blank namely the same diluent (HCl) should be prepared and kept at the same condition simultaneously.

4.5.2 Base hydrolysis

Place 0.050 g of 1-AHH into a 50 mL volumetric flask, dissolve in the 40 mL 0.1 M NaOH, sonicate for 10 minutes, allow solution to cool to the room temperature, add 0.1 M NaOH up to the mark and mix. *The concentration of solution is 1.0 mg/mL. (1-AHH stock solution BH)*

Transfer 1-AHH stock solutions into the head space vials and close them. Keep sample at ambient temperature and analyze by HPLC in 3 hours, 6 hours and 24 hours.

After the expiry of the exposure time take 1-AHH stock solution and transfer 4 mL into 10 mL volumetric flask, dilute with buffer solution up to the mark and mix. Transfer 5 mL into 10 mL volumetric flask, dilute with diluent. *The concentration of this solution is 0.20 mg/mL.* Filter solution through a syringe filters with pore size diameter of 0.22 μm, discarding first few ml of the filtrate *(*1-AHH test solution BH).

Note: The stressed blank namely the same diluent (0.1 M NaOH) should be prepared and kept at the same condition simultaneously.

4.5.3 Oxidation

Place 0.050 g of 1-AHH into a 50 mL volumetric flask, dissolve in the 40 mL 30% H_2O_2, sonicate for 10 minutes, allow solution to cool to the room temperature, add 30% H_2O_2 up to the mark and mix. *The concentration of solution is 1.0 mg/mL. (1-AHH stock solution Oxi)*

Transfer 1-AHH stock solutions into the head space vials and close them. Keep sample at ambient temperature and analyze by HPLC in 24 hours and in 7 days.

After the expiry of the exposure time take 1-AHH stock solutions and transfer 4 mL into 20 mL volumetric flask, dilute with sample diluent up to the mark and mix. Filter solution through a syringe filters with pore size diameter of 0.22 μm, discarding first few ml of the filtrate. *The concentration of this solution is 0.20 mg/mL (1-AHH test solution Oxi)*

Note: The stressed blank namely the same diluent (30% H_2O_2) should be prepared and kept at the same condition simultaneously.

4.5.4 Photolytic

Solid

Put on a thin layer of the 1-AHH powder of about 3 mm in a Petri dish and keep in Suntest XLS Tabletop Sunlight Exposure System at least 2x the ICH exposure length.

After the expiry of the exposure time place 0.050g of 1-AHH into a 50 mL volumetric flask, dissolve in the 40 mL of sample diluent, sonicate for 10 minutes, and allow solution to cool to the room temperature, add the same diluent up to the mark and mix. *The concentration of solution is 1.0 mg/mL. (1-AHH stock solution* PH_{solid}*)*

Transfer 4 mL of 1-AHH stock solution into 20 mL volumetric flask, dilute with sample diluent up to the mark and mix. *The concentration of this solution is 0.20 mg/mL.* Filter solution through a syringe filters with pore size diameter of 0.22 μm, discarding first few ml of the filtrate. (1-AHH test solution PH_{solid})

AHH solution 1 mg/ml in water in transparent glass vials with air and with nitrogen

Place 0.050 g of 1-AHH into a 50 mL volumetric flask, dissolve in H_2O, sonicate for 10 minutes, allow solution to cool to the room temperature, add the water up to the mark and mix. *The concentration of solution is 1.0 mg/mL. (1-AHH stock solution PH)*

Divide 1-AHH stock solutions in two parts, transfer into the head space vials.

Fill with nitrogen the one head space vial. The second vial just close.

Analyze the samples by HPLC up to the 56 hours (2 x the ICH exposure length).

After the expiry of the exposure time take corresponding 1-AHH stock solutions, transfer 4 mL into 20 mL volumetric flask, dilute with sample diluent up to the mark and mix. *The concentration of this solution is 0.20 mg/mL.* Filter solution through a syringe filters with pore size diameter of 0.22 μm, discarding first few ml of the filtrate. (1-AHH test solution PH_A)

Note: The stressed blank (with nitrogen and without) namely the same diluent (H_2O) should be prepared and kept at the same condition simultaneously.

4.5.5 Thermal hydrolysis

Solution 1 mg/ml in water at 50°C in closed vials with air and with nitrogen

Place 0.050 g of 1-AHH into a 50 mL volumetric flask, dissolve in the 40 mL H_2O, sonicate for 10 minutes, allow solution to cool to the room temperature, add the same solution up to the mark and mix. *The concentration of solution is 1.0 mg/mL. (1-AHH stock solution T)*

Transfer 1-AHH stock solutions into the eight head space vials, four of them fill with nitrogen, then close. The other four just close. Keep samples at 50°C and analyze by HPLC in 24 hours and in 7, 14 and 21 days.

After the expiry of the exposure time take 1-AHH stock solutions and transfer 4 mL into 20 mL volumetric flask, dilute with sample diluent up to the mark and mix. Filter solution through a syringe filters with pore size diameter of 0.22 μm, discarding first few ml of the filtrate.

The concentration of this solution is 0.20 mg/mL (1-AHH test solution T)

Note: The stressed blank namely the same diluent (H_2O) should be prepared and kept at the same condition simultaneously.

Solid at 50°C in opened vial with air and closed vial with nitrogen

Put on the 1-AHH powder (about 0.5 g) in a head space vials and keep at 50°C, analyze the samples by HPLC in 7 and 14 days.

Put on the 1-AHH powder (about 0.5 g) in a head space vials fill with nitrogen, close and keep at 50°C up to the 7 and 14 days.

After the expiry of the exposure time take the sample, place 0.050g of 1-AHH into a 50 mL volumetric flask, dissolve in the 40 mL of sample diluent, sonicate for 10 minutes, allow solution to cool to the room temperature, add the same diluent up to the mark and mix. *The concentration of solution is 1.0 mg/mL. (1-AHH stock solution T_{solid})*

Transfer 4 mL 1-AHH stock solutions into 20 mL volumetric flask, dilute with sample diluent up to the mark and mix. *The concentration of this solution is 0.20 mg/mL.* Filter solution through a syringe filters with pore size diameter of 0.22 μm, discarding first few ml of the filtrate. (1-AHH test solution T_{solid})

4.5.6 Thermal / Humidity (solid) 75% RH

Put on the 1-AHH powder (about 0.5 g) in a head space vials and keep in humidity chamber at 75% RH up to the 7 and 14 days.

After the expiry of the exposure time take the sample, place 0.050 g of 1-AHH into a 50 mL volumetric flask, dissolve in the 40 mL of sample diluent, sonicate for 10 minutes, allow solution to cool to the

room temperature, add the same diluent up to the mark and mix. *The concentration of solution is 1.0 mg/mL. (1-AHH stock solution TH)*

Transfer 4 mL 1-AHH stock solutions into 20 mL volumetric flask, dilute with sample diluent up to the mark and mix. *The concentration of this solution is 0.20 mg/mL.* Filter solution through a syringe filters with pore size diameter of 0.22 μm, discarding first few ml of the filtrate. (1-AHH test solution TH)

4.6 CALCULATIONS

Normalization method

The area normalization method will be used for calculation of content degradation product (in % of sum of the all peaks on chromatogram), i.e. assume that the sum of the areas of the peaks on chromatogram corresponding to Aminohydantoine is equal to 100%.

The degradation products will be determined at 200 nm.

Calculation of the relative percentage of each product degradation will by using the formula:

% product degradation = area of peak x 100 / sum of peak areas

Mass balance

Mass balance shows adequacy of the method by adding the assay value and the amounts of impurities and degradants the closest to 100% of the unstressed assay value (6). Amount of decomposed substances should be approximately equal to amount of formed impurities. Mass balance is calculated for confirmation of proper estimation of degree of degradation for each experiment, it reveals possible appearance of peaks of degradant hidden under the main peak and affirms there were no significant impurities neglected.

Mass balance = % assay + % sum of all degradants

Assay

Assay of AHH in stressed samples is calculated by using unstressed samples as reference when:

A_{test} - area of peak due to Aminohydantoine in the test solution

$A_{reference}$ - area of peak due to Aminohydantoine in reference solution

$$\%assay = \frac{A_{test}}{A_{reference}} \times 100$$

Content of each impurity and sum of degradation products are expressed in percent of Aminohydantoine in test solution.

Assay of Aminohydantoine in test solution is expressed in percent in relation to content of Aminohydantoine in reference solution.

Peak purity

It is important to know peak purity because co-eluting compounds could be present. Peak purity is established by using spectral information from a diode array detector. Thresholds should be set such that co-eluting peaks can be detected (6). The peak purity of Aminohydantoine has to be evaluated at the all types of forced degradation and at the all stages of it. The estimation of peak purity is performed by using software Empower 3.0 (Waters). Peak purity is confirmed by comparing the purity angle with the purity threshold (PDA Result Table). The peak is considered “pure” if the purity angle is lower than purity threshold.

Acceptance criteria

The level of degradation of AHH should be in the range from 5% to 20 %.

The value of the mass balance can be acceptable from 95 to 105 %.

The peak of AHH in the test solution has to be pure at the all types and at the all stages of forced degradation.

5. DISCUSSION AND RESULTS

5.1 ACID HYDROLYSIS (AH)

Exposing the sample to previously recommended 1 M HCl resulted into almost immediate degradation, less than one hour. The calculation of mass balance was too high (more than 105%) and the degradation was too big so it was decided to use weaker concentration of acid to provide milder conditions. The HPLC results after one hour using 0,1 M HCl showed unknown impurity with **RRT 1.20**. After longer period of time the area in chromatogram of the same RRT increased. The decrease in area of AHH could be seen. After 21 hours AHH has degraded by 10% and this was considered a sufficient degradation and further stress testing with acid was stopped.

Results after 1 hour

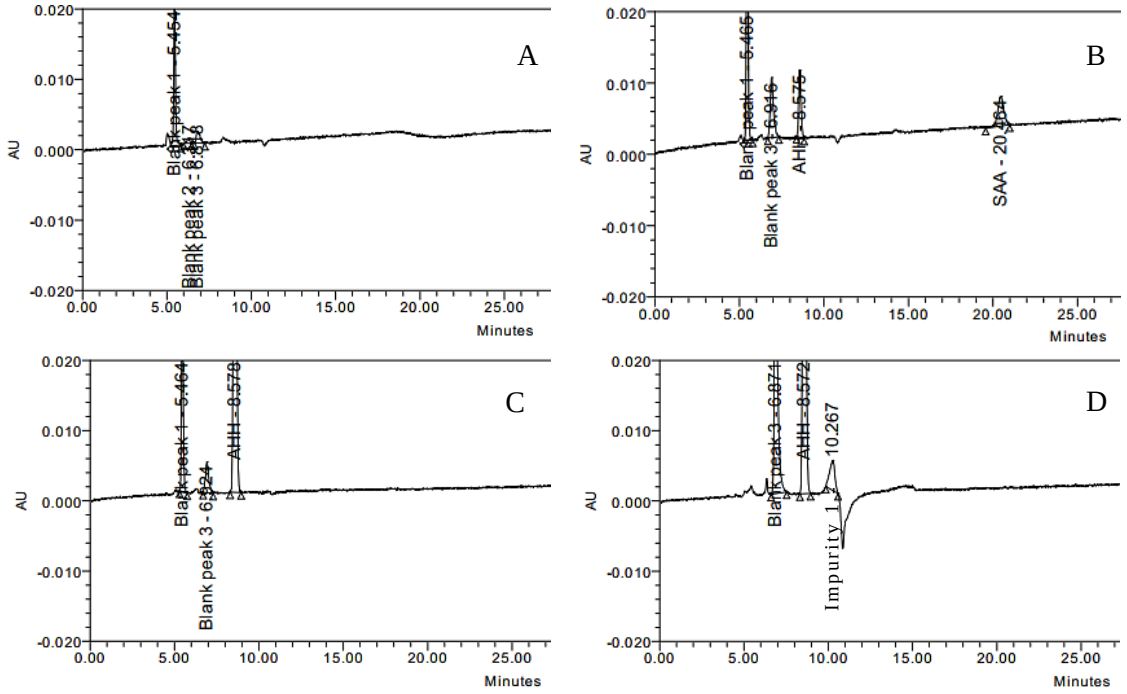
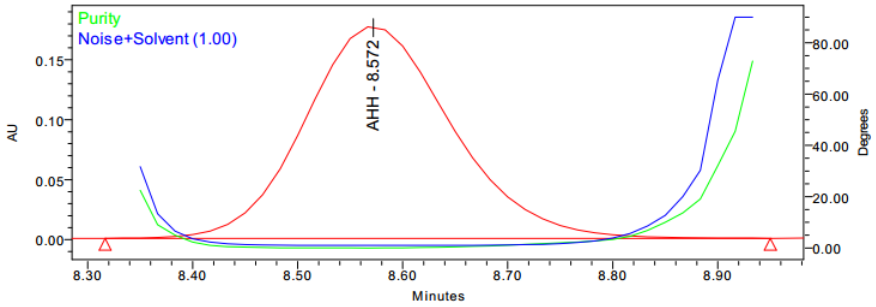


Figure 9: Chromatograms of AH 0,1N HCl 1 hour – stressed blank (A), resolution (B), reference (C), sample (D)

Peak name	RT	Area	RRT	Sum	% Degrad	% Assay	Mass Balance
AHH	8.572	1727945		1819765		99,28	104,32
Unknown impurity 1	10.267	91820	1.20		5,05		
AHH reference		*1740535*					

Table 6: AH 0,1 M HCl 1 hour



AHH	
Purity angle	0,279
Purity threshold	1,090

PDA Result Table 1:

AH - Sample 0,1 M HCl 1 hour

Figure 11: Purity plot of AHH

After 21 hours

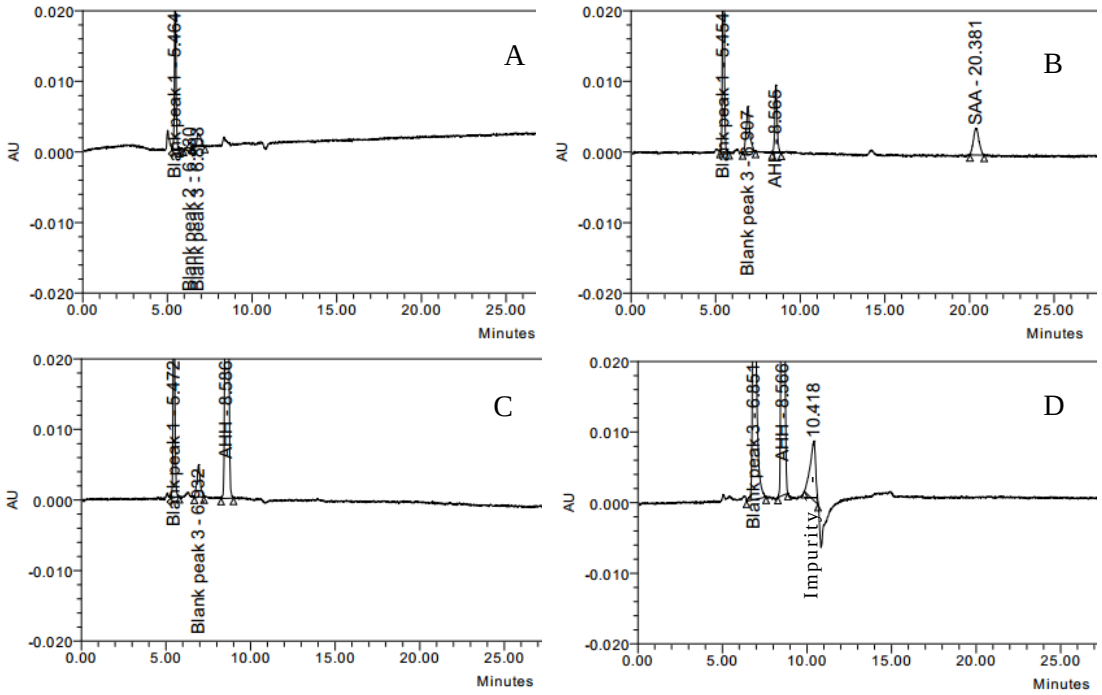


Figure 12: Chromatograms of AH 0,1N HCl 21 hours – stressed blank (A), resolution (B), reference (C), sample (D)

Peak name	RT	Area	RRT	Sum	% Degrad	% Assay	Mass Balance	Purity angle	Purity threshold
AHH	8.566	1703030		1894173		95,20	105,29	0,087	1,086
Unknown impurity 1	10.418	191143	1.22		10,09				
AHH reference		*1788844*							

Table 7: AH – 0,1 M HCl 21 hours

Time [h]	AHH Assay	Impurity 1 degradation [%]	Mass Balance
1	99,28	5,05	104,32
2,5	98,45	5,41	103,86
21	95,20	10,09	105,29

Table 8: Results of Acid hydrolysis

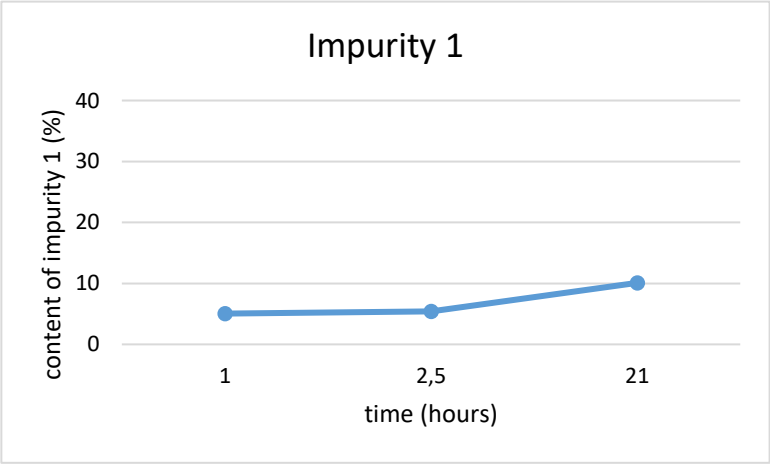


Figure 14: Change of content of unknown impurity 1

5.2 BASE HYDROLYSIS (BH)

Exposing the sample to 0,1 M NaOH resulted in one impurity with RRT 2.4 after 3 hours. Its peak matches with **SAA** through PDA result table. After 6 hours AHH degraded more and the bigger peak of SAA appeared. The stress testing was stopped after 24 hours when the degradation exceeded the limit and it showed 33% of degradation.

After 3 hours

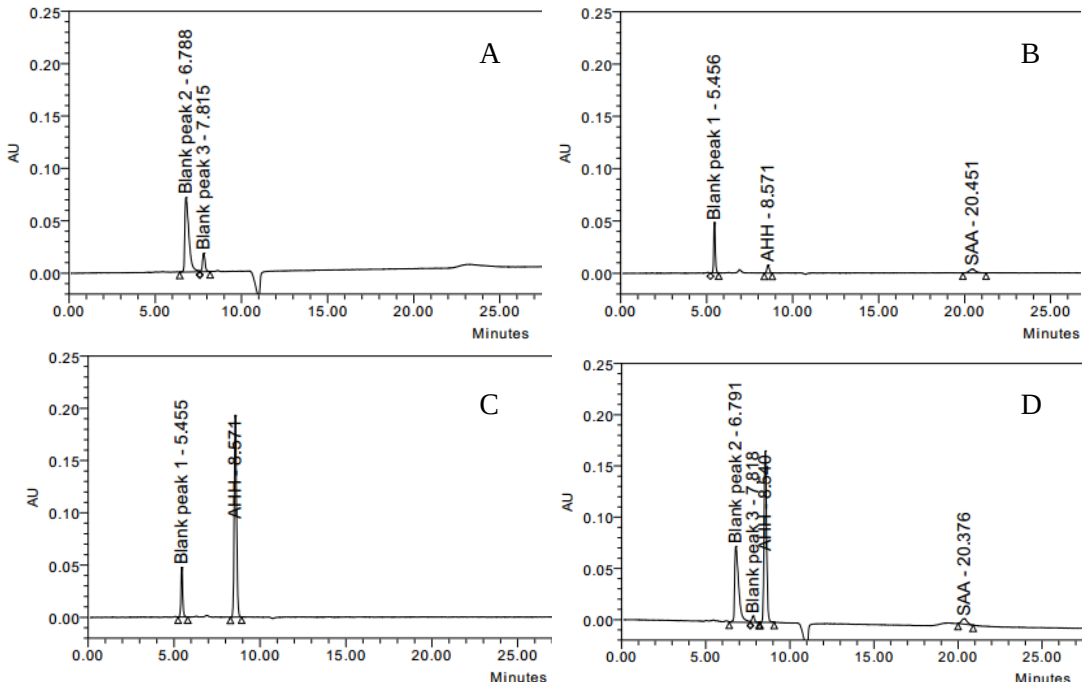
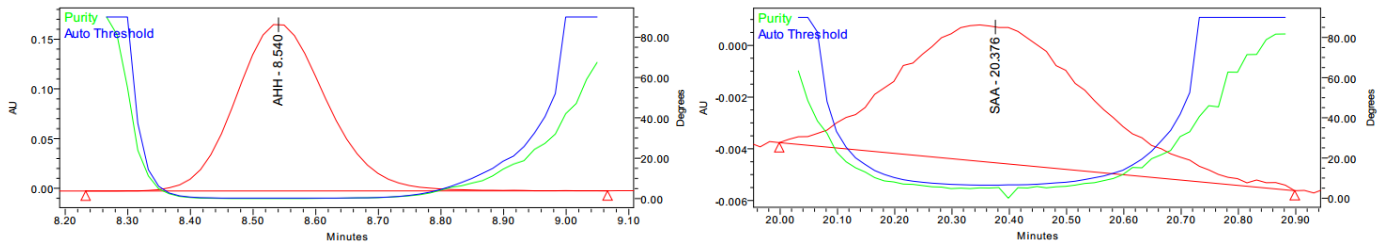


Figure 15: Chromatograms of BH 0,1N NaOH 3 hours – stressed blank (A), resolution (B), reference (C), sample (D)

Peak name	RT	Area	RRT	Sum	% Degrad	% Assay	Mass Balance	Purity angle	Purity threshold
AHH	8.540	1710609		1835303		95,16	101,95	0,130	0,301
SAA	20.376	124694	2.39		6,79			8,591	9,352
AHH reference		*1797632*							

Table 9: BH – 0,1 M NaOH 3 hours



Purity Plot 5: AHH (a) and SAA (b) – BH 0,1N NaOH 3 hours

After 24 hours

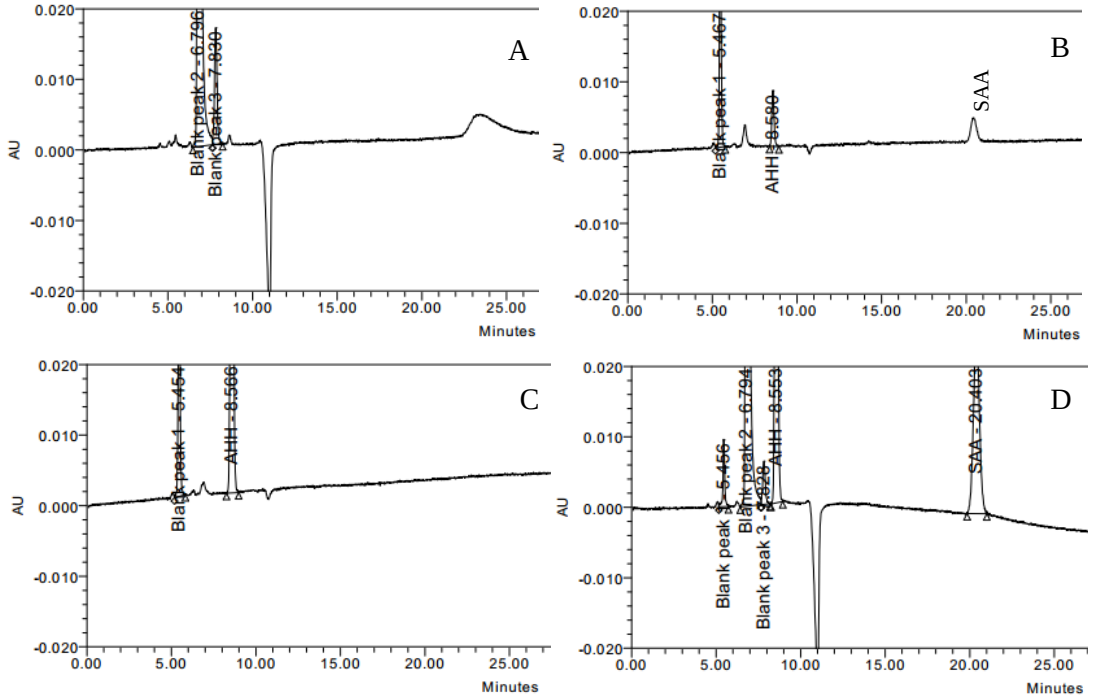


Figure 17: Chromatograms of BH 0,1N NaOH 24 hours – stressed blank (A), resolution (B), reference (C), sample (D)

Peak name	RT	Area	RRT	Sum	% Degrad	% Assay	Mass Balance	Purity angle	Purity threshold
AHH	8.553	1266954		1907189		71,58	105,15	0,160	0,324
SAA	20.40	640235	2.39		33,57			2,167	2,291
AHH reference		*1770084*							

Time [h]	AHH Assay [%]	SAA degradation [%]	Mass Balance
3	95,16	6,79	101,95
6	87,59	11,86	99,45
24	71,58	33,57	105,15

Table 10: Results of Base Hydrolysis

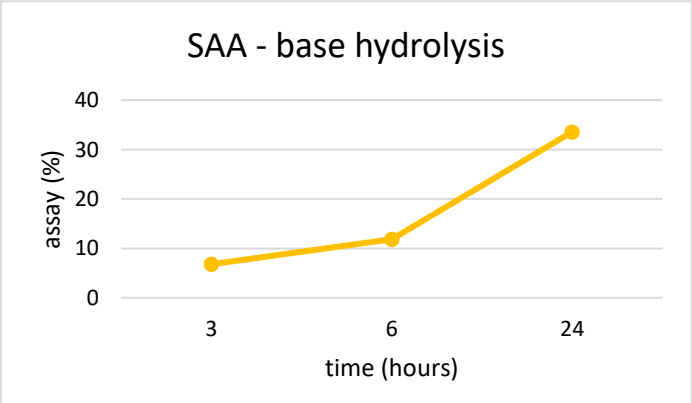


Figure 18: Content of SAA during base hydrolysis

Comparison of AHH content during acid and base hydrolysis

As can be seen in Figure 19 below, when compared the degradation of AHH caused by base hydrolysis is faster and more pronounced than during acid hydrolysis.

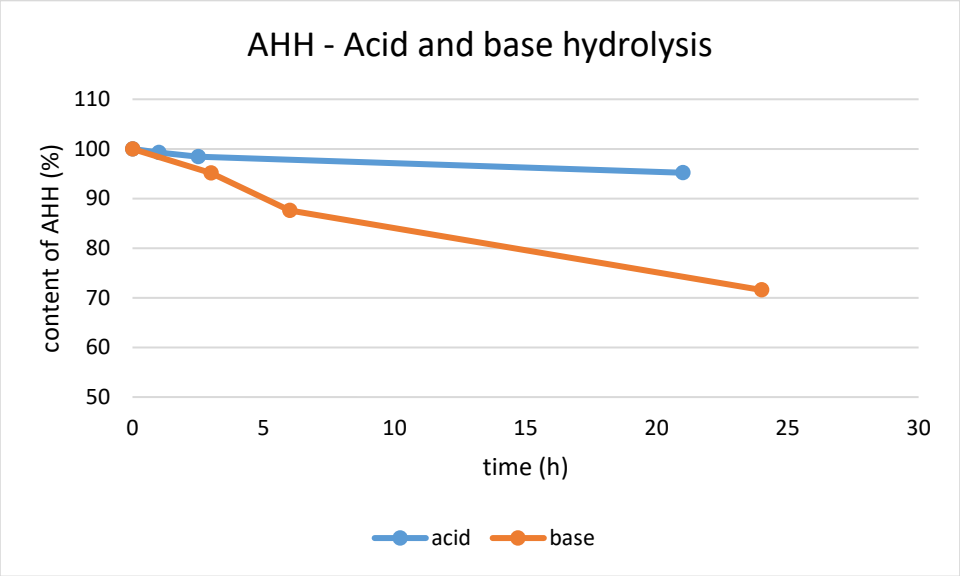


Figure 19: Graph showing content of AHH during acid (blue) and base hydrolysis (orange)

5.3 OXIDATION

The purity angles of AHH peaks in the sample and stressed blank were above the purity threshold which shows they were not pure. This happened because peaks of hydrogen peroxide and AHH were in the same position and so mass balance could not be calculated. As hydrogen peroxide is the most recommended oxidizing agent in stress testing and there was no impurity appearing in neither chromatograms after 24 hours nor 7 days it was decided it is not important to change the method.

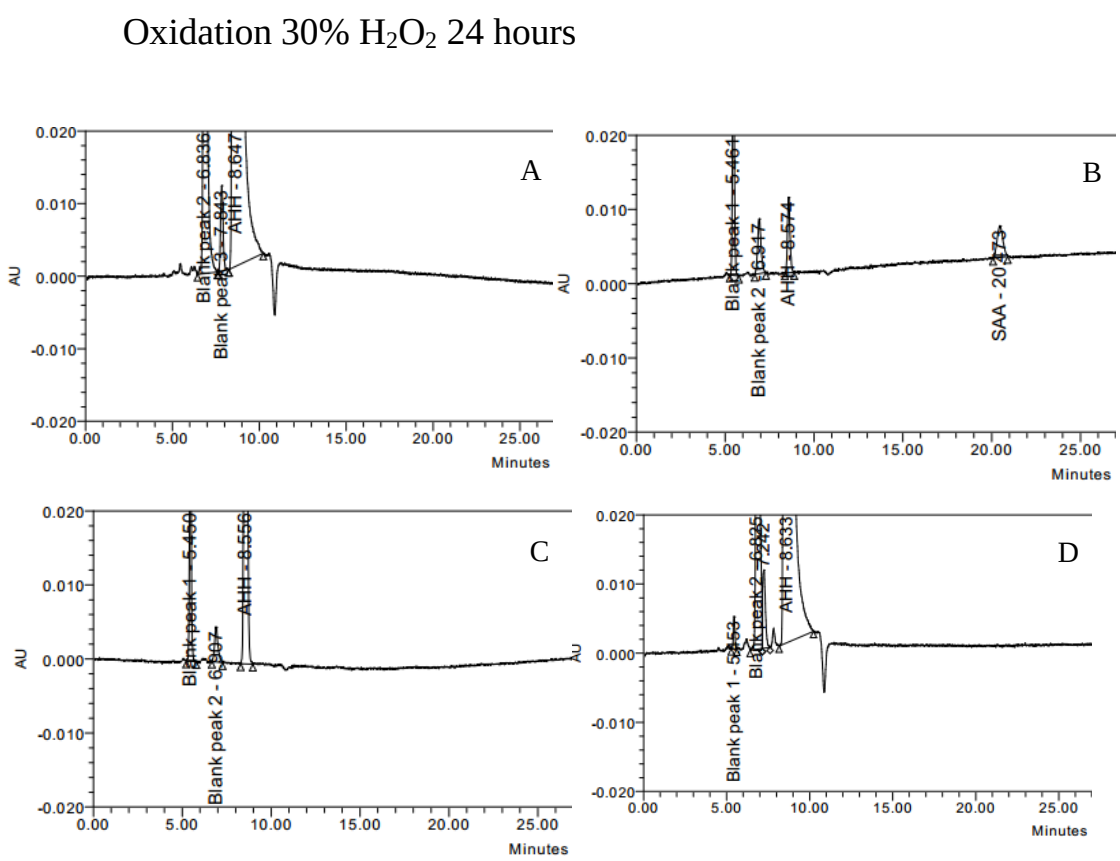
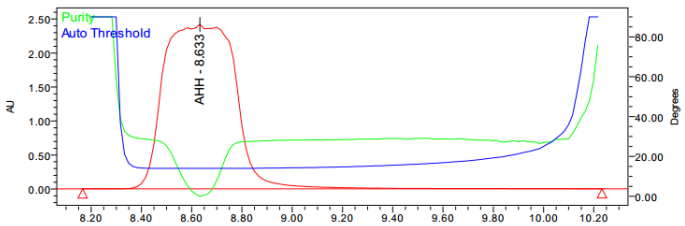


Figure 21: Chromatograms of Oxidation 30% H_2O_2 24 hours – stressed blank (A), resolution (B), reference (C), sample (D)

Peak name	RT	Area	Sum	% Assay	Mass Balance
AHH	8.633	48034604	48166094	-	-
AHH reference		*1794234*			

Table 11: Oxidation 30% H_2O_2 24 hours



Purity Plot 8: AHH - Oxidation 30% H_2O_2 24 hours

AHH	
Purity angle	11,272
Purity threshold	14,062

PDA Result Table 2: Oxidation 30% H_2O_2 24 hours

Oxidation 30% H_2O_2 7 days

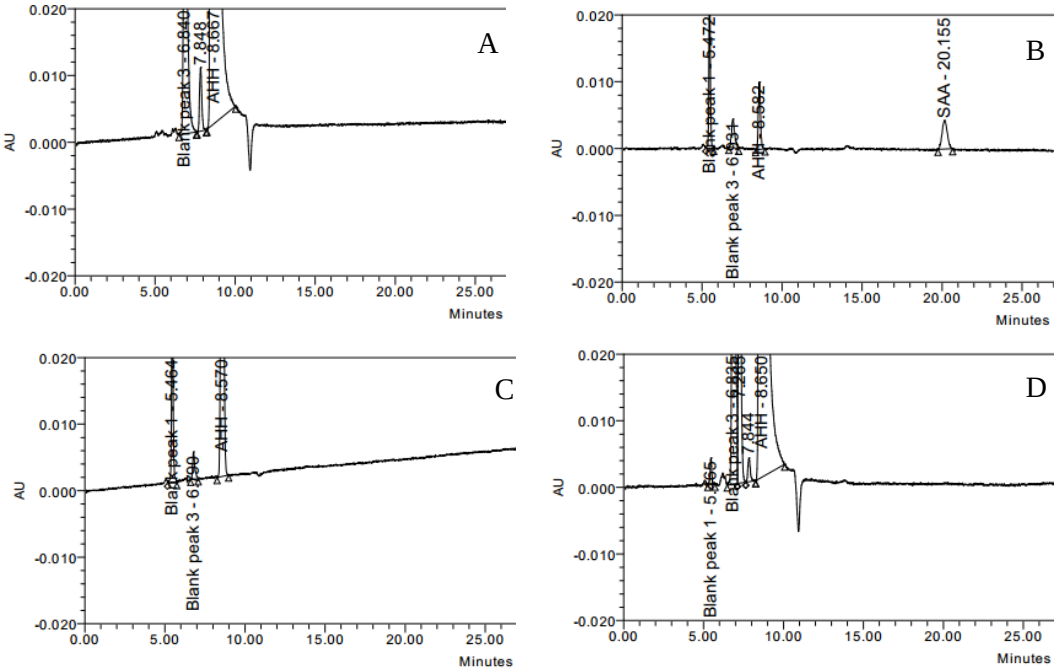
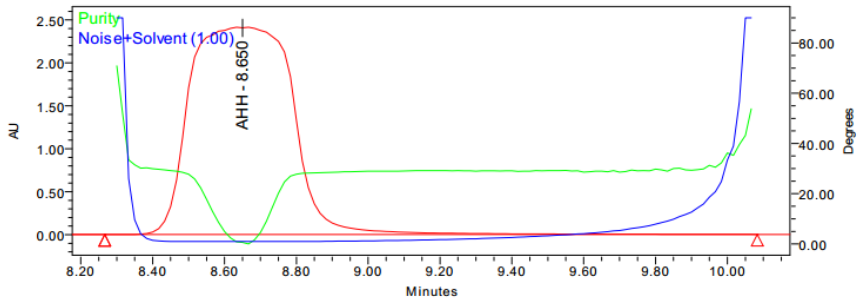


Figure 22: Chromatograms of Oxidation 30% H_2O_2 7 days – stressed blank (A), resolution (B), reference (C), sample (D)

Peak name	RT	Area	Sum	% Assay	Mass Balance
AHH	8.650	47647540	48164243	-	-
AHH reference		*1744230*			

Table 12: Oxidation 30% H_2O_2 7 days



*Purity Plot 9: **AHH - Oxidation** 30% H_2O_2 7 days*

AHH	
Purity angle	11,358
Purity threshold	1,004

PDA Result Table 3: Oxidation 30% H_2O_2 7 days

5.4 PHOTOLYTIC

After exposing the solid sample to light there was no degradation seen. After exposing solution samples to light in presence of both only air and only N_2 there was no impurity appearing. The sufficient degradation was not achieved in any case. The purity angle of AHH was under the threshold.

Solid

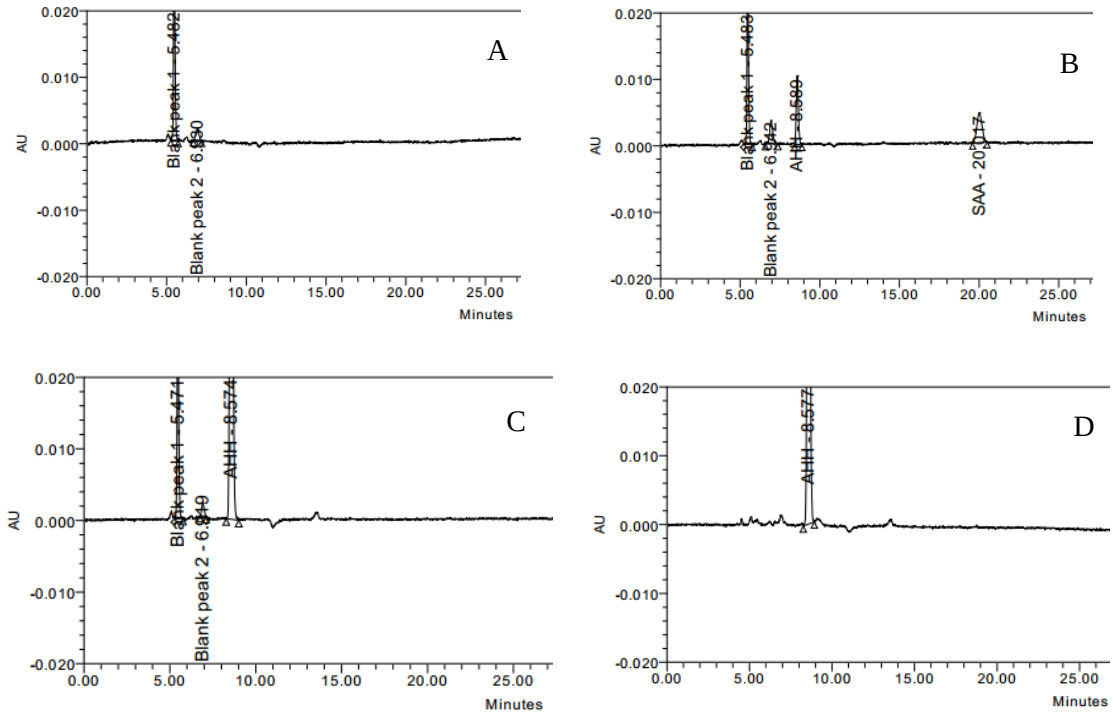
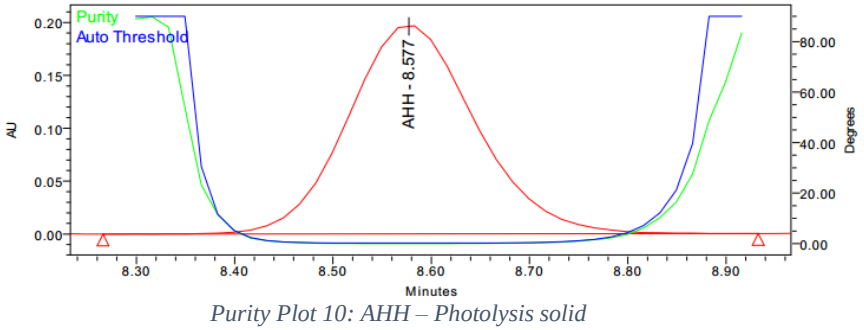


Figure 23: Chromatograms of Photolysis – solid – diluent (A), resolution (B), reference (C), sample (D)

Peak name	RT	Area	% Degrad	% Assay	Mass Balance	Purity angle	Purity threshold
AHH	8.57	1790838	-	99,00	99,00	0,119	0,296
AHH reference		*1808913*					

Table 13: Photolysis solid



Purity Plot 10: AHH – Photolysis solid

Solution – Air

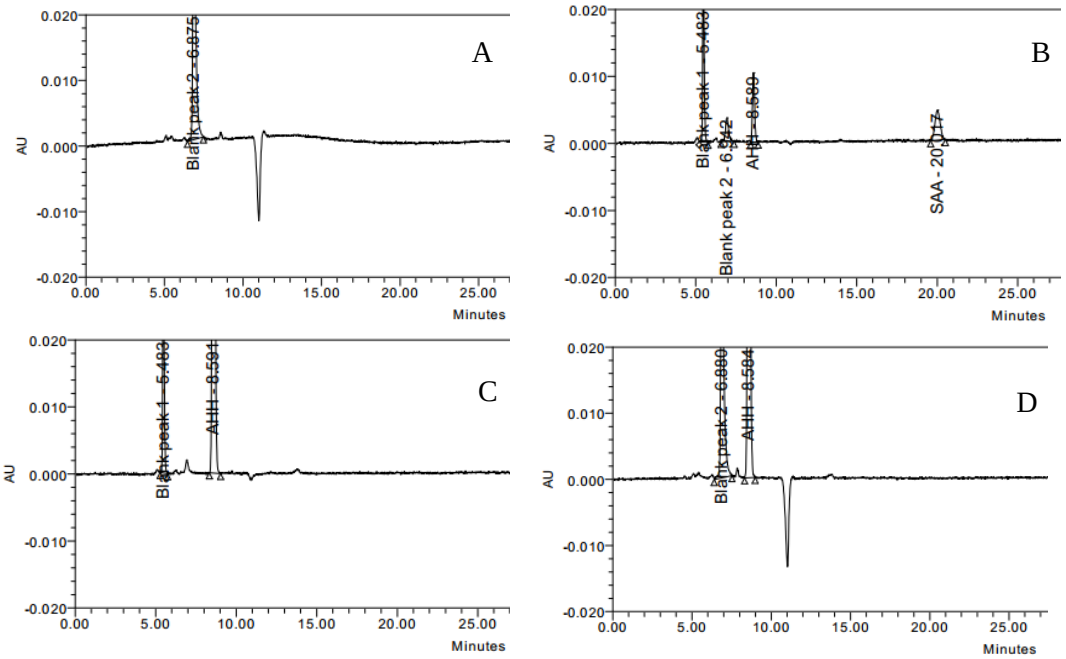
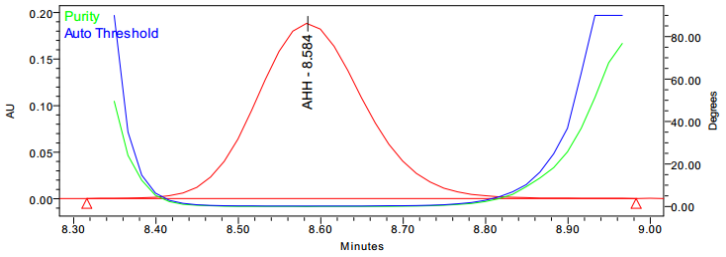


Figure 24: Chromatograms of Photolysis solution in air – stressed blank (A), resolution (B), reference (C), sample (D)

Peak name	RT	Area	% Degrad	% Assay	Mass Balance	Purity angle	Purity threshold
AHH	8.584	174694	-	96,81	96,81	0,101	0,295
AHH reference		*180442*					

Table 14: Photolysis solution in air



Purity Plot 11: AHH – Photolysis solution in air

Solution - N_2

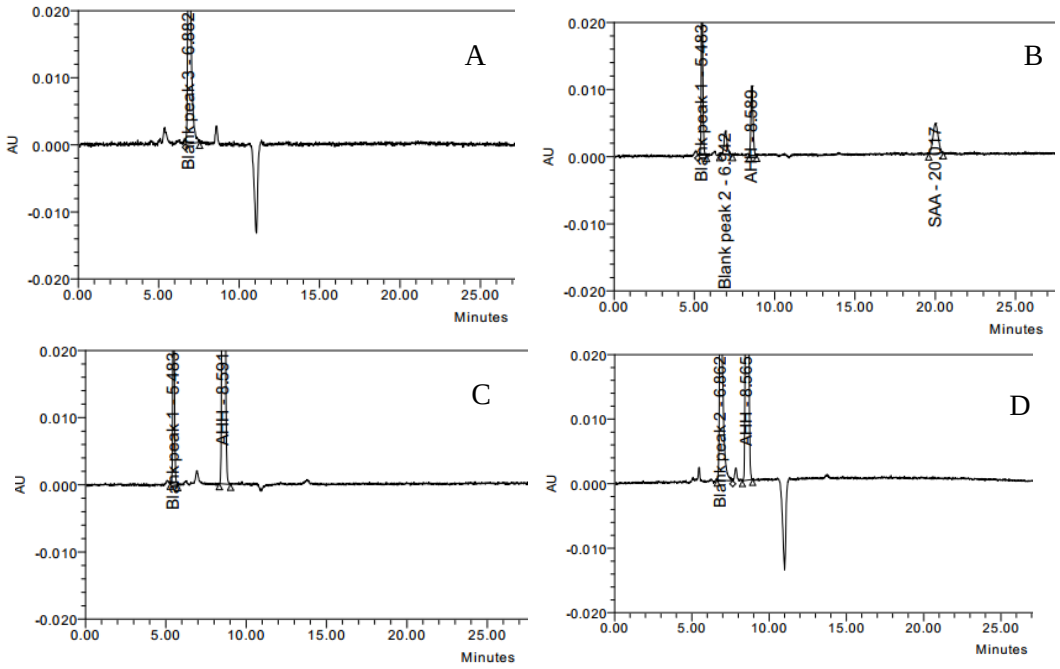
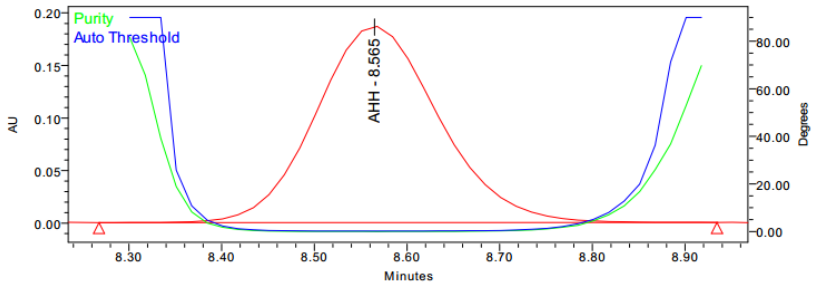


Figure 25: Chromatograms of Photolysis solution in N_2 – stressed blank (A), resolution (B), reference (C), sample (D)

Peak name	RT	Area	% Degrad	% Assay	Mass Balance	Purity angle	Purity threshold
AHH	8.565	173179	-	95,98	95,98	0,098	0,292
AHH reference		*180442*					

Table 15: Photolysis solution in N_2



Purity Plot 12: AHH – Photolysis solution in N_2

5.5 THERMAL HYDROLYSIS – SOLUTION

After 24 hours of exposing solution samples with air and N_2 to 50°C there was no degradation seen and no significant degradants appearing. After 7 days a small peak of SAA appeared with 1,06% (N_2) and 2,06% (air) of degradation. In 14 and 21 days samples were tested and they showed growing amount of SAA. After 21 days there was also unknown impurity with **RRT** around **1.60** (Impurity 4) found in both reactions.

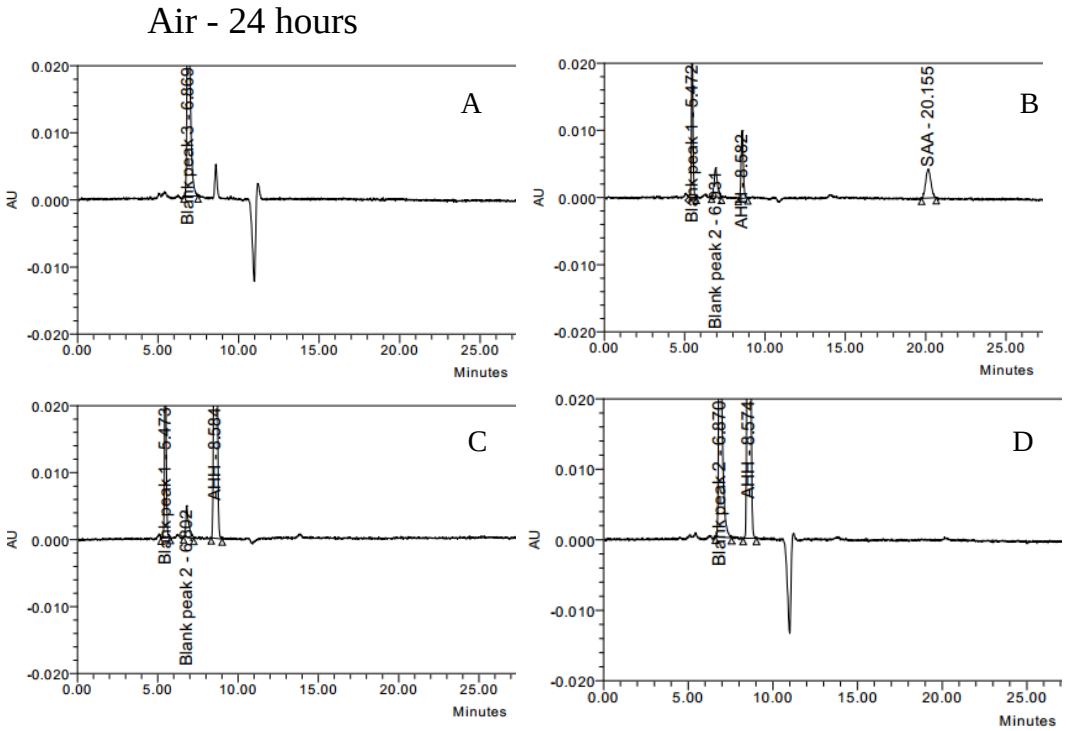
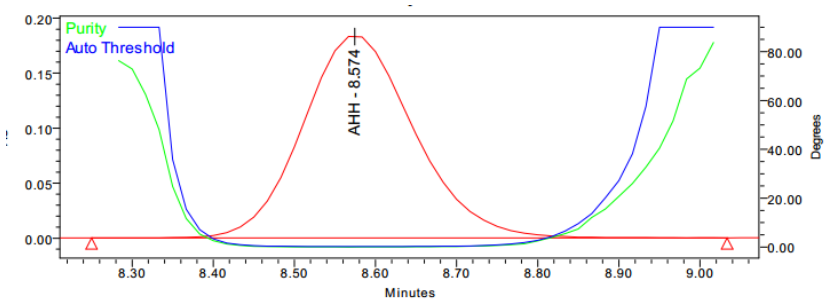


Figure 26: Thermal hydrolysis – Solution Air 24 hours - stressed blank (A), resolution (B), reference (C), sample (D)

Peak name	RT	Area	% Degrad	% Assay	Mass Balance	Purity angle	Purity threshold
AHH	8.57	176283	-	97,03	97,03	0,125	0,299
AHH reference		*181455*					

Table 16: Thermal hydrolysis – Solution Air 24 hours



Purity Plot 13: AHH - Thermal hydrolysis – Solution Air 24 hours

Air - 21 days

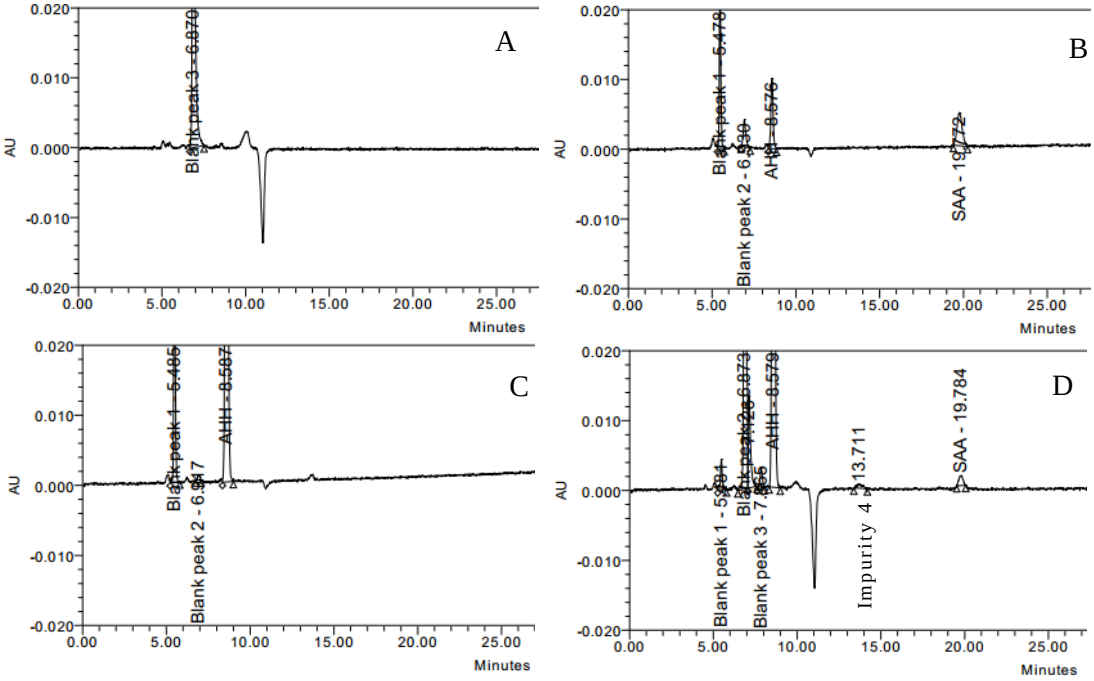
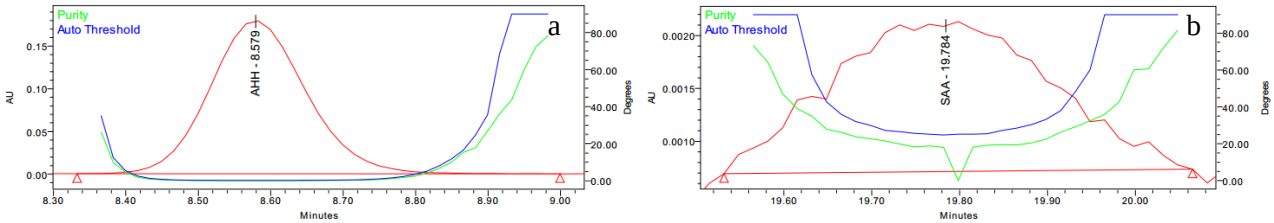


Figure 27: Thermal hydrolysis – Solution Air 21 days - stressed blank (A), resolution (B), reference (C), sample (D)

Peak name	RT	Area	RRT	Sum	% Degrad	Sum of degradants	% Assay	Mass Balance	Purity angle	Purity threshold
AHH	8.579	1669122			-	-	96,32		0,115	0,307
Unknown impurity 4	13.711	14359	1.60	1708476	0,84	2,3		98,63		
SAA	19.784	24995	2.31		1,46				25,961	32,324
AHH reference		*1769558*								

Table 17: : Thermal hydrolysis – Solution Air 21 days



Purity Plot 14: AHH (a) and SAA (b) – Thermal hydrolysis – Solution Air 21 days

N_2 - 24 hours

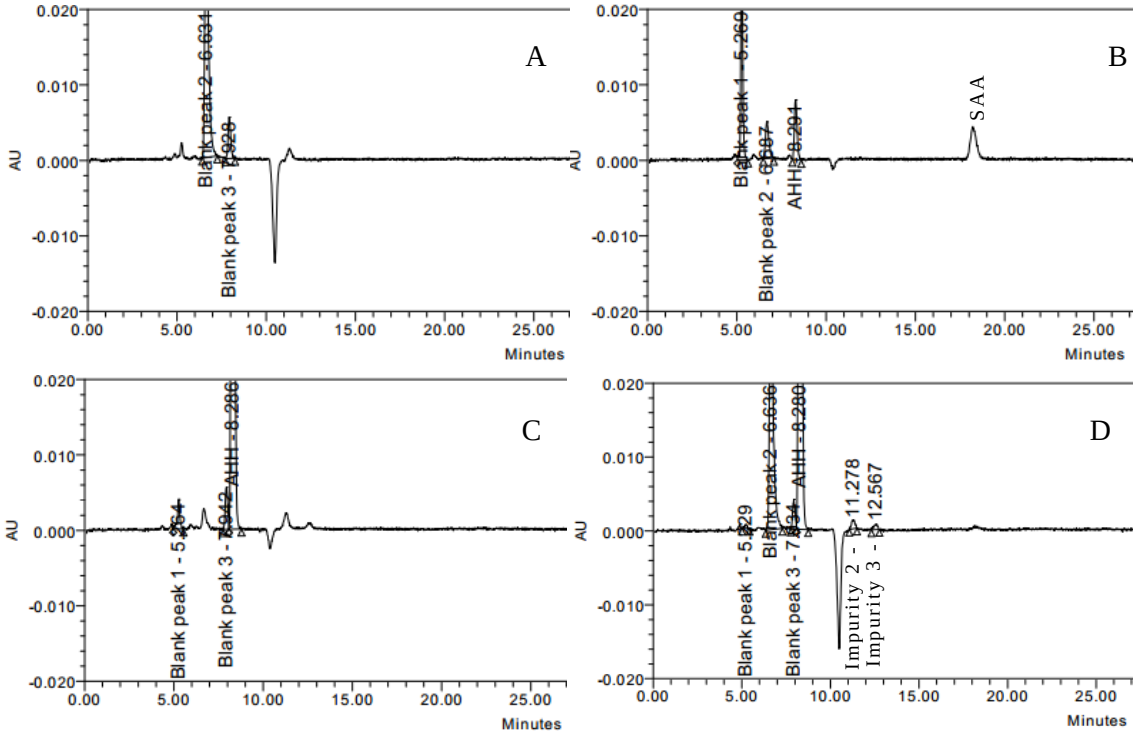
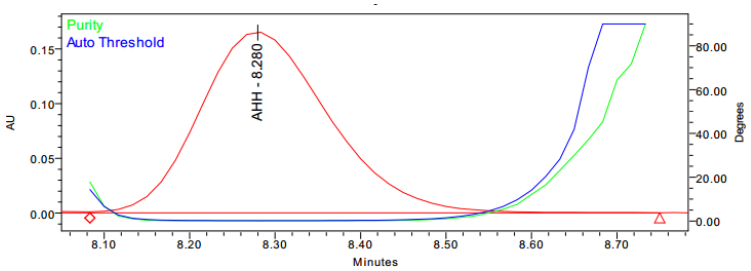


Figure 28: Thermal hydrolysis – Solution N_2 24 hours - stressed blank (A), resolution (B), reference (C), sample (D)

Peak name	RT	Area	RRT	Sum	% Degrad	Sum of degradants	% Assay	Mass Balance	Purity angle	Purity threshold
AHH	8.280	1737123		1764657	-	-	99,56	101,12	0,105	0,301
Unknown impurity 2	11.278	16407	1.36		0,93	1,56				
Unknown impurity 3	12.567	11127	1.52		0,63					
AHH reference		*1744740*								

Table 18: Thermal hydrolysis – Solution N2 24 hours



Purity Plot 15: AHH - Thermal hydrolysis – Solution N_2 24 hours

N_2 - 21 days

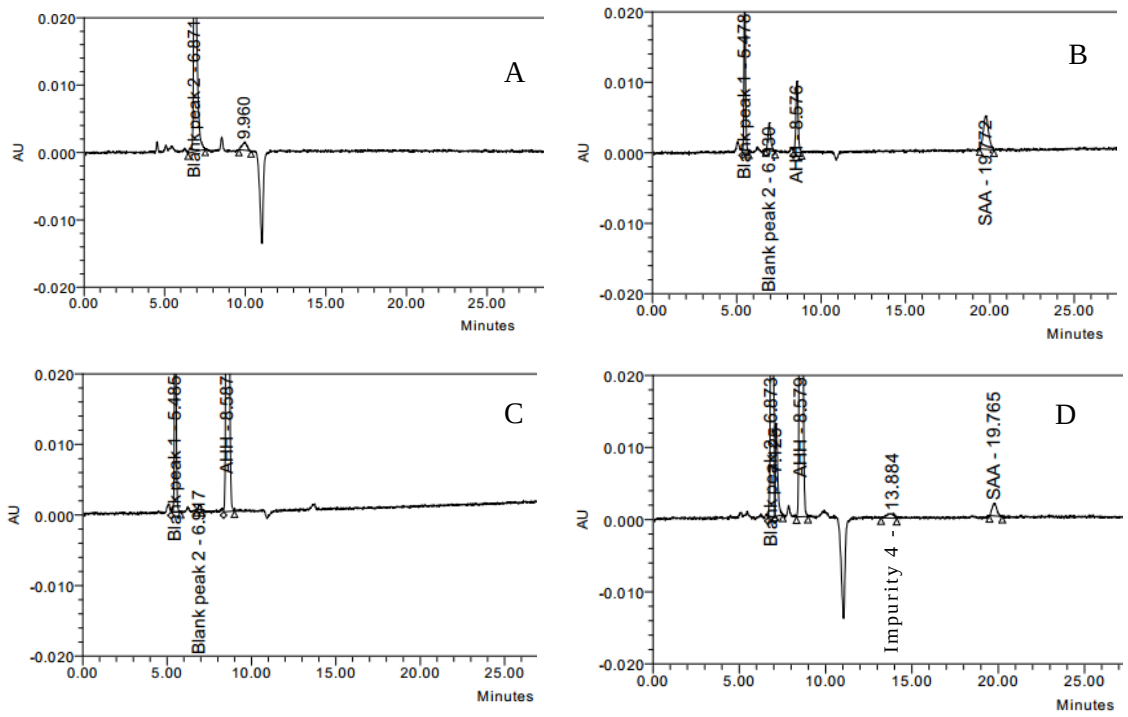
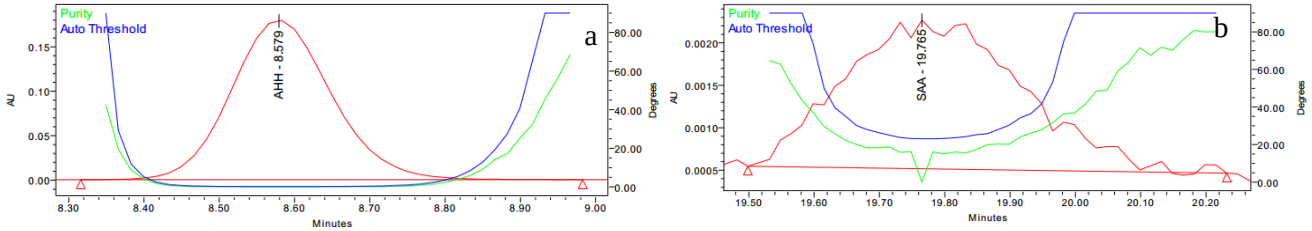


Figure 34: Thermal hydrolysis – Solution N_2 21 days - stressed blank (A), resolution (B), reference (C), sample (D)

Peak name	RT	Area	RRT	Sum	% Degrad	Sum of degradants	% Assay	Mass Balance	Purity angle	Purity threshold
AHH	8. 579	1675974		1729088	-	-	96,72	99,79	0,103	0,306
Unknown impurity 4	13.884	19676	1.62		1,14	2,07				
SAA	19.765	33438	2.30		1,93				26,165	33,754
AHH reference		*1732803*								

Table 19: Thermal hydrolysis – Solution N_2 21 days



Purity Plot 21: AHH (a) and SAA (b) – Thermal hydrolysis – Solution N_2 21 days

Comparison of AHH during thermal hydrolysis with solution in air and in nitrogen

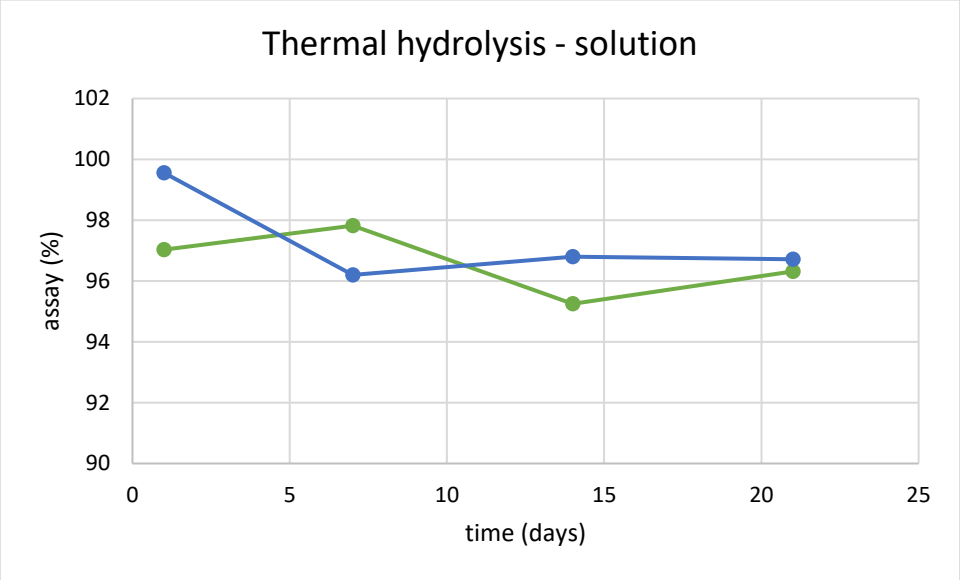


Figure 35: Graph showing changes of content of AHH after thermal hydrolysis in air (green) and N_2 (blue)

Comparison of SAA during thermal hydrolysis with solution in air and in nitrogen

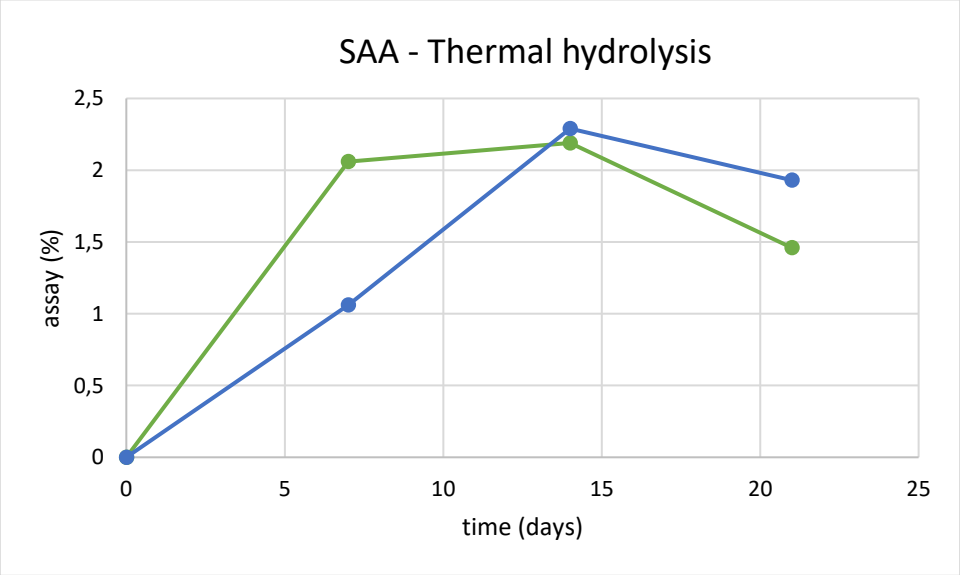


Figure 36: Graph showing appearance of content of SAA after thermal hydrolysis in air (green) and N_2 (blue)

5.6 THERMAL HYDROLYSIS – SOLID

After 7 days of exposing sample with air and with N_2 to 50°C there was no impurity found and AHH showed no degradation. After 14 days there was still no sign of degradation. The substance remained stable in 50°C in air and N_2.

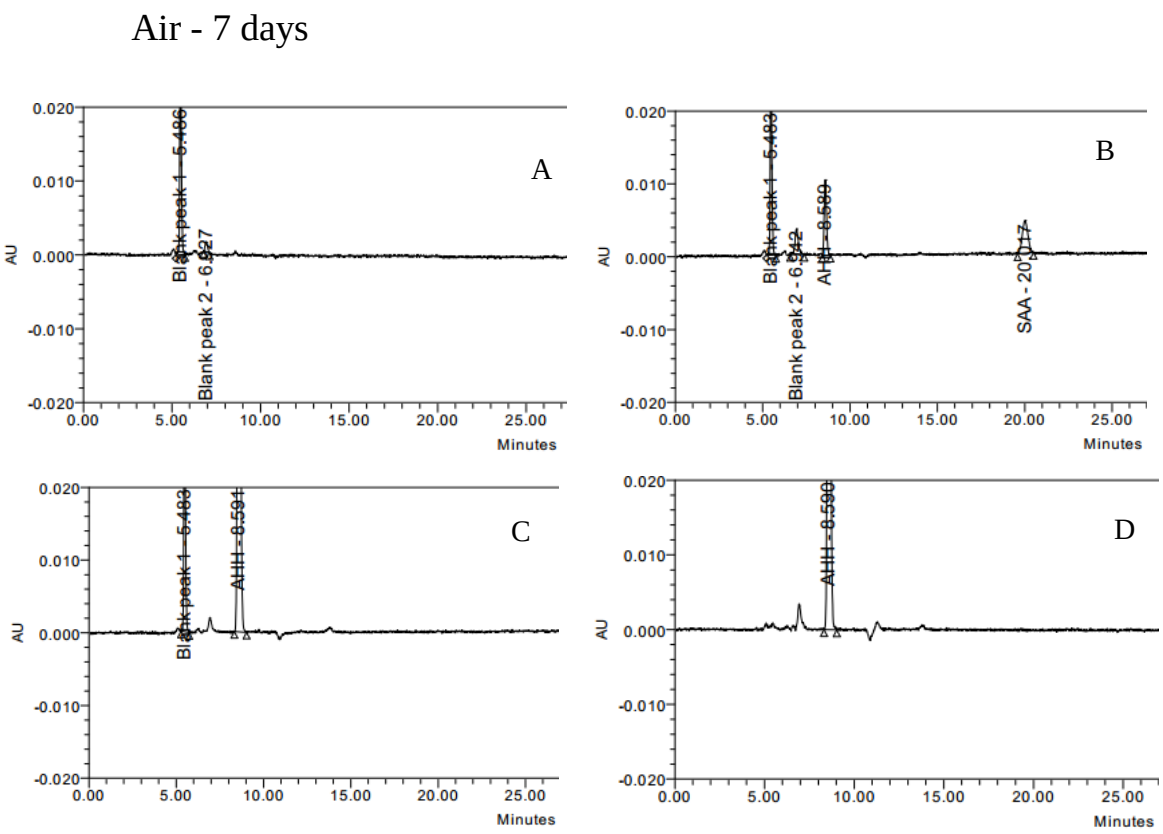
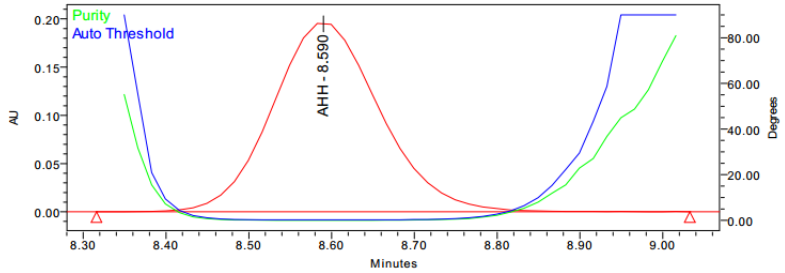


Figure 37 : Thermal hydrolysis – Solid air 7 days – diluent (A), resolution (B), reference (C), sample (D)

Peak name	RT	Area	% Degrad	% Assay	Mass Balance	Purity angle	Purity threshold
AHH	8.590	1780749	-	98,69	98,69	0,105	0,302
AHH reference		*1804423*					

Table 20: Thermal hydrolysis – Solid air 7 days



Purity Plot 22: AHH - Thermal hydrolysis – Solid air 7 days

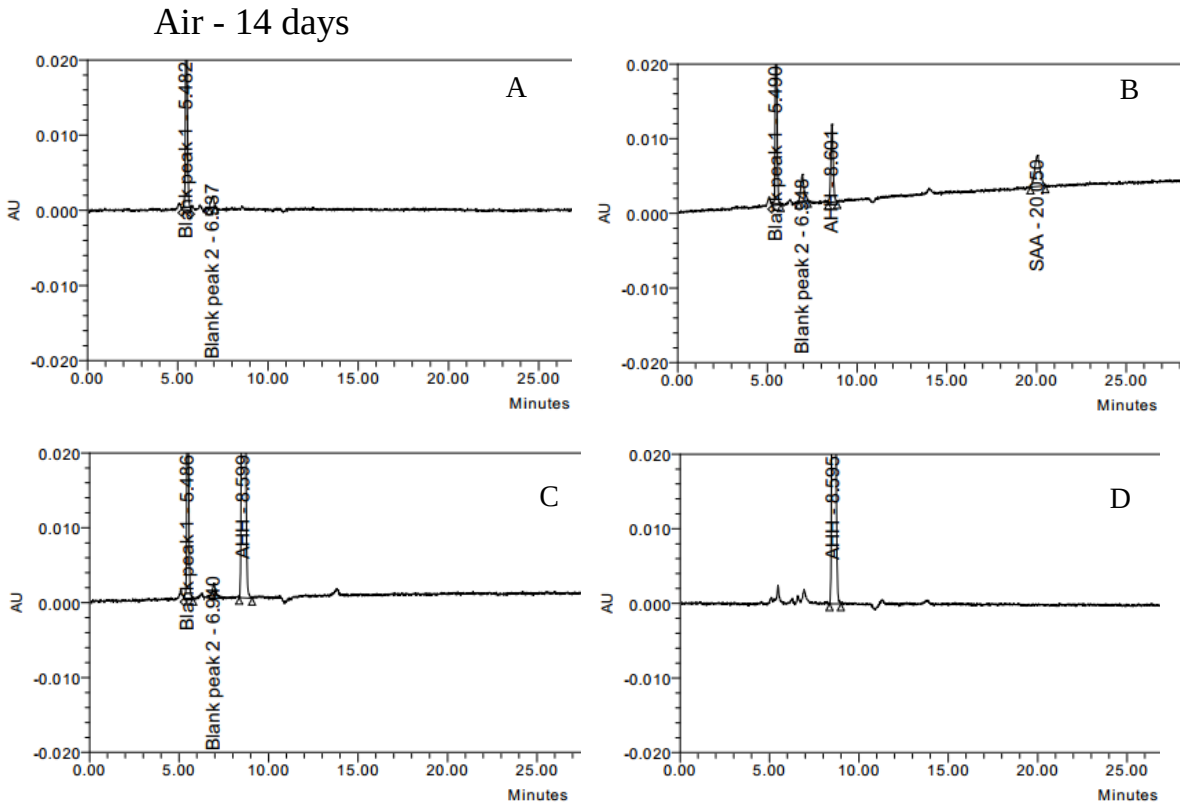


Figure 38: Thermal hydrolysis – Solid air 14 days - diluent (A), resolution (B), reference (C), sample (D)

Peak name	RT	Area	% Degrad	% Assay	Mass Balance	Purity angle	Purity threshold
AHH	8.595	1779318	-	98,00	98,00	0,100	0,292
AHH reference		*1815639*					

Table 21: Thermal hydrolysis – Solid air 14 days

N_2 - 7 days

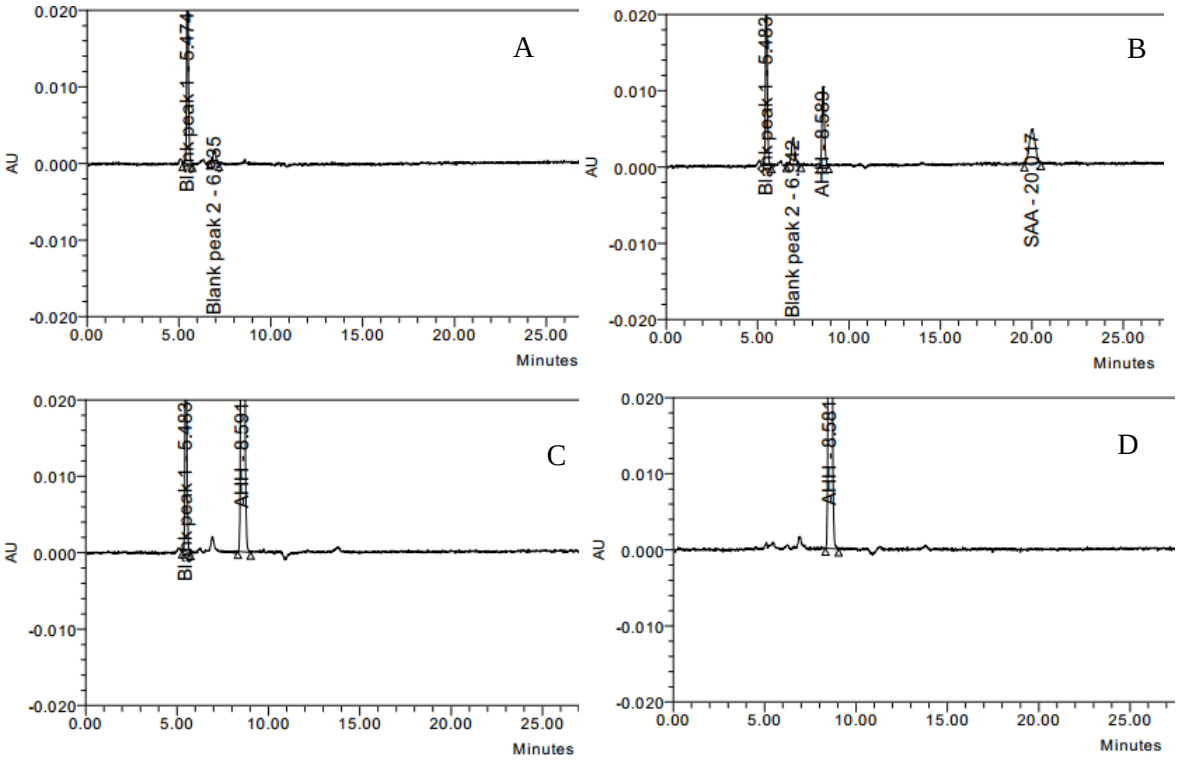
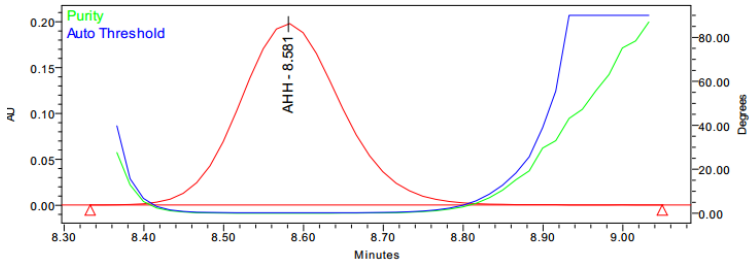


Figure 39: Thermal hydrolysis – Solid N_2 7 days - diluent (A), resolution (B), reference (C), sample (D)

Peak name	RT	Area	% Degrad	% Assay	Mass Balance	Purity angle	Purity threshold
AHH	8.581	1788870	-	99,14	99,14	0,107	0,306
AHH reference		*1804423*					

Table 22: Thermal hydrolysis – Solid N_2 7 days



Purity Plot 24: AHH - Thermal hydrolysis – Solid N_2 7 days

N_2 - 14 days

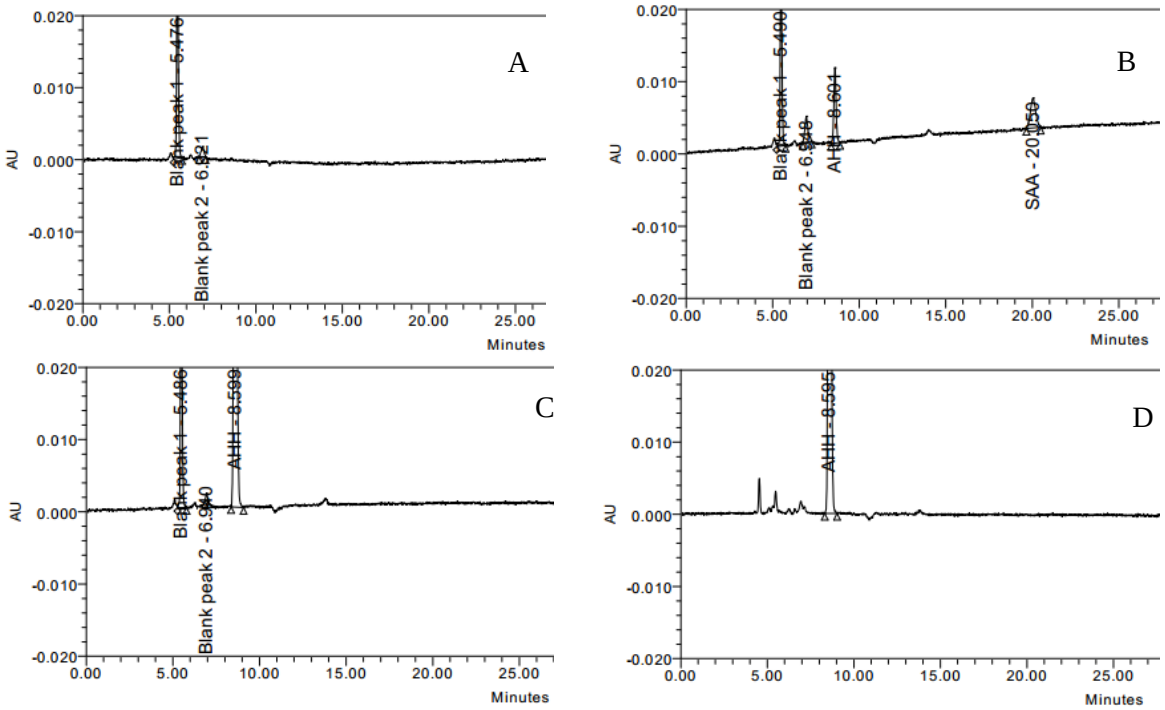


Figure 41: Thermal hydrolysis – Solid N_2 14 days - diluent (A), resolution (B), reference (C), sample (D)

Peak name	RT	Area	% Degrad	% Assay	Mass Balance	Purity angle	Purity threshold
AHH	8.595	1806152	-	99,48	99,48	0,105	0,312
AHH reference		*1815639*					

Table 23: Thermal hydrolysis – Solid N_2 14 days

5.7 THERMAL / HUMIDITY

After 7 days of exposing the sample to heat and humidity there was no appearance of significant degradation. This situation remained the same after 14 days. The purity angle of AHH was under the threshold.

7 days

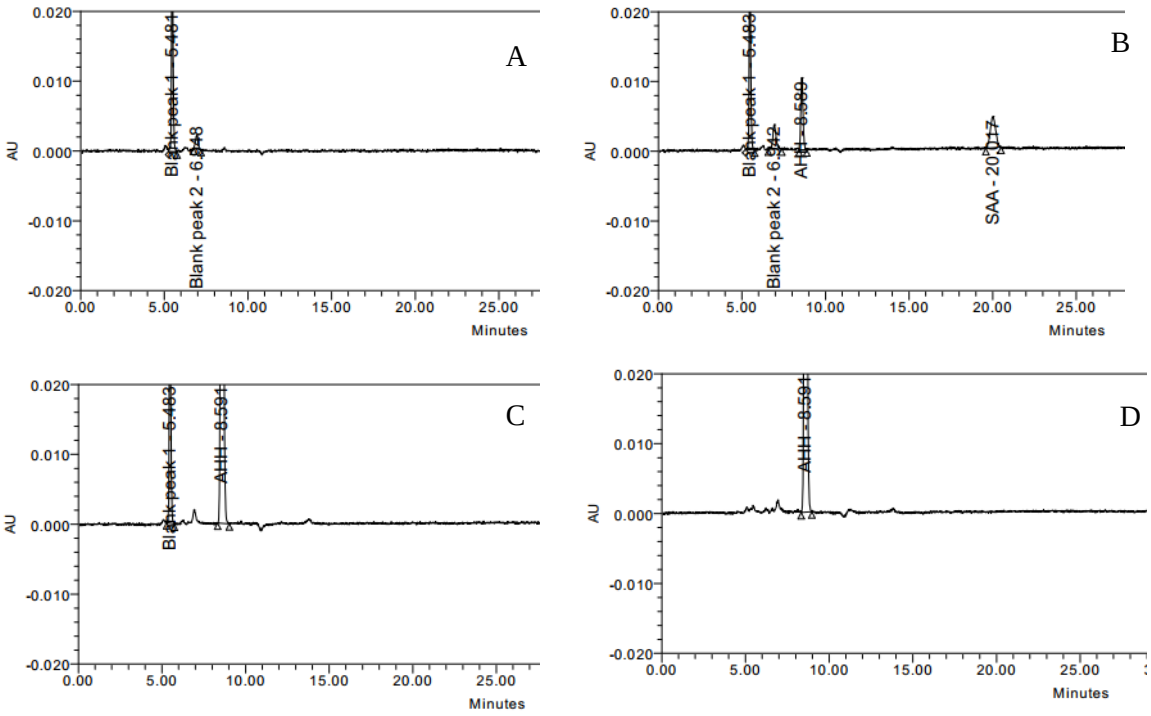
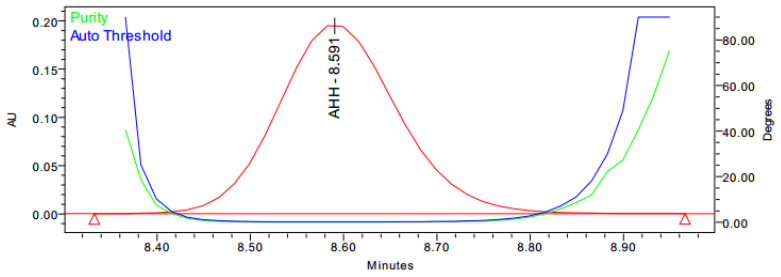


Figure 42: : Thermal + Humidity 7 days - unstressed blank (A), resolution (B), reference (C), sample (D)

Peak name	RT	Area	% Degrad	% Assay	Mass Balance	Purity angle	Purity threshold
AHH	8.591	1776518	-	98,45	98,45	0,095	0,297
AHH reference		*1804423*					

Table 24: Thermal + Humidity 7 days



Purity Plot 27: AHH - Thermal + Humidity 7 days

14 days

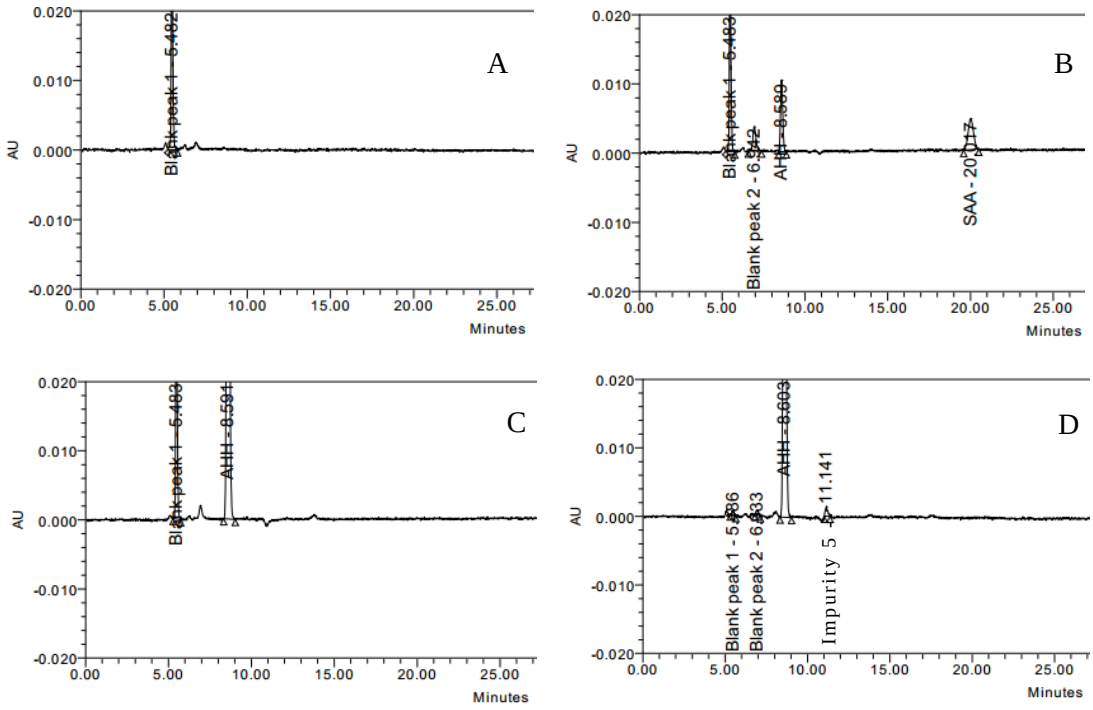


Figure 43: Thermal + Humidity 14 days - unstressed blank (A), resolution (B), reference (C), sample (D)

Peak name	RT	Area	RRT	Sum	% Degrad	% Assay	Mass Balance	Purity angle	Purity threshold
AHH	8.603	1721247		1735198	-	95,39	96,19	0,105	0,303
Unknown impurity 5	11.141	13951	1.30		0,80				
AHH reference		*1804423*							

Table 25: Thermal + Humidity 14 days

6. CONCLUSION

The final results of stress study on AHH are summarized in Table 26. All of previously planned stress tests were finished. SAA (known degradation product) and some other unknown impurities (1, 2, 3, 4, and 5) were obtained as a product of various stress studies. The substance did not reach sufficient level of degradation (5 – 20% of API) (6) (27) in some cases - thermal (solid), thermal/humidity (solid) and photolytic degradation. In most of undertaken tests the level of degradation was according to the decrease of assay under the limit. However ICH announces that "studies may be terminated after an appropriate exposure level" (5). During base hydrolysis the level of degradation reached 33,6 % after 24 hours. This indicates the time period was too long to achieve sufficient degradation with 0,1 M NaOH.

Peak purity of AHH was evaluated by comparing purity angle to purity threshold. In all cases apart from oxidation stress testing after 7 days the purity angle was below purity threshold hence there was no impurity hiding inside the peak. This means the peak of AHH was pure. In oxidation stress study after 24 hours the purity angle was below the threshold but the purity plot spectrum showed the process of the purity angle curve was at some part above the threshold so the peak cannot be considered completely pure even at this time of oxidation reaction. Comparing the result of sample to the stressed blank and reference revealed that the reason is a peak of hydrogen peroxide hiding inside AHH peak.

The mass balance was supposed to not exceed the limit from 95 to 105% at all stages of forced degradation to confirm the suitability of analytical method as all the impurities were detected. The amount of decomposed substances should be approximately equal to amount of formed impurities and there should be no second degradants appearing. The mass balance was calculated showing results from the lowest 95,98% (photolytic reaction of solution in nitrogen) to the highest 105,15% (base hydrolysis) and 105,29% (acid hydrolysis) being even slightly above the limit. As the various sources claim different extent of the range it can be concluded that the mass balance was acceptable at all stages of the study.

On the basis of the results of this research which are concluded in the Table 26, the substance can be classified according to the classification system of Saranjit Singh (28) as labile in acidic and alkaline conditions. It proved to be stable in tests of photostability, thermal hydrolysis and combination of heat and humidity. Oxidation stress studies showed the substance to be practically stable but the method and used condition were not suitable for AHH. The study can be repeated under different conditions to get more accurate results.

Stress type			Time	Degradant	Assay [%]	Sum of degradants [%]	Mass balance [%]	Peak purity of AHH
Acid hydrolysis			1 h	1	99,28	5,05	104,32	PA: 0,279 PT: 1,090
			2,5 h		98,45	5,41	103,86	PA: 0,205 PT: 1,100
			21 h		95,20	10,09	105,29	PA: 0,087 PT: 1,086
Base hydrolysis			3 h	SAA	95,16	6,79	101,95	PA: 0,130 PT: 0,301
			6 h		87,59	11,86	99,45	PA: 0,119 PT: 0,304
			24 h		71,58	33,57	105,15	PA: 0,160 PT: 0,324
Oxidation			24 h	-				PA: 11,272 PT: 14,062
			7 days					PA: 11,358 PT: 1,004
Photolytic	solid		2x ICH	-	99,00	-	99,00	PA: 0,119 PT: 0,296
	solution	air			96,81		96,81	PA: 0,101 PT: 0,295
		N_2			95,98		95,98	PA: 0,098 PT: 0,292
Thermal hydrolysis	solution	air	24 h	-	97,03	-	97,03	PA: 0,125 PT: 0,299
		N_2		2; 3	99,56	1,56	101,12	PA: 0,105 PT: 0,301
		air	7 days	SAA	97,82	2,06	99,88	PA: 0,184 PT: 1,163
		N_2			96,20	1,06	97,26	PA: 0,112 PT: 0,306
		air	14 days		95,25	2,19	97,43	PA: 0,130 PT: 1,139
		N_2			96,80	2,29	99,10	PA: 0,119 PT: 1,123
		air	21 days	SAA; 4	96,32	2,3	98,63	PA: 0,115 PT: 0,307
		N_2			96,72	2,07	99,79	PA: 0,103 PT: 0,306
	solid	air	7 days	-	98,69	-	98,69	PA: 0,105 PT: 0,302
		N_2			99,14		99,14	PA: 0,107 PT: 0,306
		air	14 days		98,00		98,00	PA: 0,100 PT: 0,292
		N_2			99,48		99,48	PA: 0,105 PT: 0,312
Thermal / humidity			7 days	-	98,45	-	98,45	PA: 0,095 PT: 0,297
			14 days	5	95,39	0,80	96,19	PA: 0,105 PT: 0,303

Table 26: Summary of results of forced degradation studies on AHH; PA = purity angle, PT = purity threshold

The developed stability indicating method determination of impurities in 1-Aminohydantoine hydrochloride can be validated and used in routine analysis for further study of the stability of 1-Aminohydantoine hydrochloride.

REFERENCES

1. **ICH.** Q1A (R2). *Stability Testing of New Drug Substances and Products.* [Online] February 06, 2003. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q1A_R2/Step4/Q1A_R2__Guideline.pdf.

2. **Kats, Michael.** Biopharm International. *Forced Degradation Studies: Regulatory considerations and implementation.* [Online] July 01, 2005. http://www.biopharminternational.com/forced-degradation-studies-regulatory-considerations-and-implementation.

3. **Hicks, Simon R. J.** Forced Degradation to Develop Stability-indicating Methods. *Pharmaceutical Outsourcing.* [Online] January 01, 2012. http://www.pharmoutsourcing.com/Featured-Articles/37640-Forced-Degradation-to-Develop-Stability-indicating-Methods/.

4. **Levin, Shulamit.** High Performance Liquid Chromatography (HPLC) in the pharmaceutical analysis. [Online] February 2010. http://www.forumsci.co.il/HPLC/WEB-Pharm_Review/HPLC_pharma_WEB_15-2-10.html.

5. **ICH.** Q1B. *Stability Testing: Photostability Testing of New Drug Substances and Products.* [Online] 1996. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q1B/Step4/Q1B_Guideline.pdf.

6. **Maheswaran, Ragine.** FDA Perspectives: Scientific Considerations of Forced Degradation Studies in ANDA Submissions. *PharmTech.com.* [Online] May 02, 2012. http://www.pharmtech.com/fda-perspectives-scientific-considerations-forced-degradation-studies-anda-submissions.

7. **Singh, Ranjit.** Current trends in forced degradation study for pharmaceutical product development. *Indian Journal of Pharmaceutical Education and Research.* June 15, 2012, Vol. 3, Issue No. 1.

8. **Reynolds, Dan W.** Available Guidance and Best Practises for Conducting Forced Degradation Studies. *Pharmaceutical Technology.* February 2002.

9. **ICH.** Q3A (R2). *Impurities in New Drug Substances.* [Online] 2006. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q3A_R2/Step4/Q3A_R2__Guideline.pdf.

10. —. Q3C (R5). *Impurities: Guideline for residual solvents.* [Online] 2011. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q3C/Step4/Q3C_R5_Step4.pdf.

11. **Blessy, M, Patel, Ruchi D. and Prajapati, Prajesh N.** Development of forced degradation and stability indicating studies of drugs. *Journal of Pharmaceutical Analysis.* 2014, Vol. 4, Issue 3.

12. **Nováková, Lucie and Douša, Michal.** *Moderní HPLC separace v teorii a praxi I.* 2013. 978-80-260-4243-3.

13. **Kok, Wim Th.** Principles of Detection. [book auth.] Elena Katz. *Chromatographic Science Series v. 78: Handbook of HPLC.* 1998.

14. **McNaught, A. D. and Wilkinson, A.** *IUPAC. Compendium of Chemical Terminology, 2nd ed. (the "Gold Book").* Oxford : Blackwell Scientific Publications, XML on-line corrected version: http://goldbook.iupac.org (2006-) created by M. Nic, J. Jirat, B. Kosata, 1997.

15. **Lingema, Henk.** Chromedia. [Online] http://www.chromedia.org/chromedia?waxtrapp=yoovpDsHonOvmOlIEcCI&subNav=yarwnEsHonOvmOlIEcCIbB.

16. **Guillarme, Davy.** HILIC: A Critical Evaluation. *LCGC.* [Online] 2014. http://www.chromatographyonline.com/hilic-critical-evaluation.

17. **Douša, Michal.** HPLC.cz. [Online] 2016. http://www.hplc.cz.

18. **Klimeš, Jiří.** *Kontrolně-analytické hodnocení léčiv lékopisnými metodami.* 2011. 978-80-87009-29-1.

19. **PerkinElmer Informatics.** *ChemDraw.* [Computer program]

20. **Reaxys.** [Online] https://www.elsevier.com/solutions/reaxys.

21. **National Center for Biotechnology information.** PubChem Compound Database. *CID=67308.* [Online] 22. Nov. 2015. https://pubchem-ncbi-nlm-nih-gov.ezproxy.is.cuni.cz/compound/67308.

22. **OlainFarm.** OlainFarm: Furazidin. [Online] http://olainfarm.lv/product/furazidin/.

23. **Drugs.com.** [Online] https://www.drugs.com/international/furazidin.html.

24. **Dybowskia, Bartosz, et al.** Ciprofloxacin and furagin in acute cystitis: comparison of early immune and microbiological results. *International Journal of Antimicrobial Agents.* 2008, Vol. 31, Issue 2.

25. **OlainFarm.** OlainFarm: Furamag. [Online] http://olainfarm.lv/product/furamags/.

26. **Latvijas Zāļu reģistrs.** [Online] https://www.zva.gov.lv/zalu-registrs/.

27. **Brummer, Dr. Hildegard.** How to approach a forced degradation study. *SGS Life Science*. January 2011, Issue No. 31.

28. **Saranjit Singh, Monika Bakshi.** Guidance on Conduct of Stress Tests to Determine Inherent Stability of Drugs. *Pharmaceutical Technology On-line.* April 2000.

29. **Hanai, T.** *HPLC : A Practical Guide.* 1999. 978-0-85404-515-0.